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Reproduction in the Rat on Purified Diets Containing Succinylsulfathiazole.*

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Nelson and Evans¹ have shown that placing normal adult female rats on purified diets deficient in pantothenic acid 2-3 weeks before mating or even as late as the day of mating resulted in impaired reproduction, *i.e.* failure of implantation, resorptions, or defective litters. The normality of the paired-fed controls receiving supplements of pantothenic acid eliminated the factors of inanition and of other possible specific dietary deficiencies as causes for these marked upsets in reproduc-

tion. One of the possible dietary deficiencies thus eliminated under the experimental conditions was that of pteroylglutamic acid, which has since been shown to be necessary for optimal lactation in the rat maintained on the above-mentioned control diet.² In the present communication we wish to report the reproductive behavior of adult females placed on a succinylsulfathiazole-containing diet (SST-diet) deficient in pteroylglutamic acid (PGA) for various periods prior to mating.

* Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: synthetic pteroylglutamic acid through the courtesy of Dr. T. H. Jukes of the Lederle Laboratories, Pearl River, New York; succinyl-sulfathiazole by Sharp and Dohme, Inc., Glenolden, Pa.; crystalline B vitamins, 2-methyl-1,4-naphthoquinone and alphatocopherol from Merck and Company, Inc., Rahway, N.J.

¹ Nelson, M. M., and Evans, H. M., *J. Nutrition*, 1946, **31**, 497.

Experimental. Normal female rats (Long-Evans strain), 3 to 4 months of age were bred with normal males and placed on the deficient or control diet the day of breeding. Other groups of rats were maintained on the deficient diet for one, two, or three months and then bred as rapidly as possible. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation has occurred; all rats were weighed at regular intervals.

² Nelson, M. M., and Evans, H. M., *Arch. Biochem.*, **13**, 265.

TABLE I.
Reproduction of Rats Maintained on Purified SST-Diets.

Time exp. diets fed	No. of rats bred	Failed implantation	Resorptions of implantations	Litters of implantations	Total No. young	Young born dead	Avg size of litter	Avg wt young
		%	%	%		%		g
Day of Mating	10	0	0	100	87	0	8.7 (3-11)	5.9
" "	12	0	0	100	112	0	9.3 (6-12)	5.5
35 Days before Mating	10	0	10	90	64	9	7.1 (2-10)	4.7
64 " " "	9	0	44	56	29	3	5.8 (3-9)	5.8
92 " " "	10	10	33	67	32	6	5.3 (2-9)	5.5

The PGA-deficient diet was composed of 24% alcohol-extracted casein, 63% sucrose, 8% hydrogenated cottonseed oil (Crisco), 4% salts,³ and 1% succinylsulfathiazole. Crystalline vitamins per kilogram diet were: 300 µg d-biotin, 5 mg 2-methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 50 mg calcium pantothenate 400 mg inositol and 1.0 g choline chloride. Control rats received the identical diet supplemented with 5.5 mg synthetic pteroylglutamic acid per kilogram diet. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 Chick Units vitamin D, 6 mg synthetic alpha-tocopherol, and 650 mg corn oil (Mazola).

Results. Table I shows that the omission of pteroylglutamic acid from the SST-purified diet on the day of breeding did not interfere with reproduction. When the deficient diet was started one month before breeding, reproduction was slightly impaired as shown by

the occurrence of resorption and of young born dead together with the slight decrease in the number of young per litter and their weight at birth. Extending the deficiency to two months before breeding resulted in marked upsets in reproductive behavior. Almost half of the animals resorbed instead of littering and the average size of the litter was markedly decreased. A further extension of the deficiency period to 3 months before breeding did not result in any greater impairment of reproduction. Additional groups of animals placed on the deficient diet at an earlier age have shown essentially the same picture.

It may be noted that no interference with implantation occurred in these PGA-deficient animals as was the case with pantothenic acid deficient animals during reproduction. A considerably longer deficiency period (1-3 months) was required to produce reproductive upsets with the SST-diet than was necessary for the pantothenic acid deficient diets (0-3 weeks). The resistance of our rats to a "folic acid" deficiency instituted after weaning has been previously pointed out.² Studies on the curative effects of pteroylglutamic

³ Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

acid after one to three months of the deficiency and on the relation of calorie restriction to these reproductive upsets on the SST-diet are in progress.

Summary. Adult rats maintained on a puri-

fied diet containing succinylsulfathiazole and supplemented with all the known vitamins except pteroylglutamic acid from one to 3 months before breeding showed impaired reproduction.

16068

Effect of Estrogens on Malic Dehydrogenase of Rat Liver.*

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The natural and synthetic estrogens are important in controlling the function of reproductive tissues, and they are also known to affect the metabolism and function of other tissues not directly concerned with reproduction. It is probable that these changes depend basically on the part played by the estrogens in certain of the enzymatic processes concerned with cellular function. As a means of obtaining information on these basic cellular processes McShan and Meyer¹ studied the effect of the estrogens on the succinoxidase system of liver and pituitary tissues. It was found that both natural and synthetic estrogens were effective inhibitors of this system, and that the inhibition was mediated through the action of the estrogens with the cytochrome oxidase of the system. This work has been extended to the study of the effect of the estrogens on the malic dehydrogenase system using the method reported by Potter² for the determination of this dehydrogenase. In addition results are given on the activity of malic dehydrogenase when the cytochrome c of the system is replaced by brilliant cresyl blue.

Experimental. Male and female rats of the

Sprague-Dawley strain which were three to four months old were used in these experiments.

The rats were killed by decapitation and the tissues were removed immediately, weighed and placed in sharp-pointed homogenizing tubes containing 0.1 ml of glass-distilled water. These homogenizing tubes containing the tissues were kept in an ice bath during homogenization and until the tissues were placed in the Warburg flasks.

The method of Potter² for the determination of malic dehydrogenase was followed in these experiments. Two and one-half per cent water homogenates of the liver tissue were used throughout. Conventional Warburg flasks without side arms were employed. The main compartment of the flasks contained, in addition to the desired amount of liver homogenate, 0.3 ml of 0.5 M l-malic acid, 0.6 ml of 0.5 M glutamic acid, 0.6 ml of 0.1 M nicotinamide, 0.3 ml of a 0.5% (5 mg per ml) solution of coenzyme I (diphosphopyridine nucleotide), 0.3 ml of 4×10^{-4} M cytochrome c, 0.4 ml of 0.2 M phosphate buffer pH 7.4, and enough water to make a final reaction volume of 3.0 ml. The center wells of the flasks contained 0.1 ml of 2N NaOH. All solutions were made with glass-distilled water.

The cytochrome c was made from beef heart muscle according to the method of Keilin and Hartree³ except that the final

* Supported in part by the Committee on Research in Endocrinology of the National Research Council, and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

¹ McShan, W. H., and Meyer, R. K., *Arch. Biochem.*, 1946, **9**, 165.

² Potter, V. R., *J. Biol. Chem.*, 1946, **165**, 311.

³ Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. (London)*, 1937, **122B**, 298.

TABLE I.
Malic Dehydrogenase Activity of Liver Homogenate.

0.15	ml of 2.5% homogenate			0.4
	0.2	0.25	0.3	
	Q _{O₂}			
	59.0	—	59.0	57.4
	81.6	—	78.8	
65.4	72.1	67.6	—	
	59.7	60.0	57.0	
	53.7	49.8	45.7	
64.4	60.9	—	—	
	106.6	101.3	—	
	90.6	93.0	—	

product was dialyzed against glass-distilled water instead of 1% sodium chloride solution. The coenzyme I was prepared in our laboratory according to the LePage modification of the Williamson and Green⁴ method of preparation. Both Eimer and Amend, and Eastman 1-malic acids were used, and values obtained with each were found to be in close agreement.

Approximately 20 minutes elapsed between the time the animals were killed and the placing of the flasks in the Warburg bath at 38°C. They were allowed to equilibrate for 6 minutes, the manometer stopcocks were closed and readings were taken at 10, 20 and 30 minutes. Q_{O₂} values were based on the average of these 3 readings.

Results and Discussion. A series of determinations was made to ascertain whether the oxygen uptake was proportional to the amount of liver homogenate used. In these runs, quantities of liver varying from 0.15 to 0.4 ml of 2.5% homogenate were used. The data presented in Table I show that the oxygen uptake of the malic dehydrogenase system is essentially proportional to the amount of liver tissue used within a narrow range of wet weight of tissue. There were several determinations made, however, in which an exact proportionality was not obtained.

Several studies were made employing various estrogenic compounds and sodium androsterone sulfate[†] as *in vitro* inhibitors of malic dehydrogenase activity of rat liver.

The test system contained 0.25 ml of a 2.5% liver homogenate and 0.15 ml of 2×10^{-3} M solution of the estrogenic compound. The estrogens employed were diethylstilbestrol, hexestrol, dienestrol and disodium 3,4-diphenylhexane-p,p'-dioxyacetate and ben-zestrol estrogenic compounds No. 020 (HO-C₆H₄-CH₂-CHC₂H₅-CH₂-C₆H₄-OH), No. 103 (HO-C₆H₄-CHCH₃-CH₂-CHC₃H₇-C₆H₄-OH) and No. 221B-2 (HO-C₆H₄-CHC₂H₅-CHC₂H₅-CHCH₃-C₆H₄-OH). These inhibitors, with the exception of disodium 3,4-diphenylhexane-p,p'-dioxyacetate, which is water soluble, were dissolved in 0.5 ml water that contained 0.03 ml of 2N NaOH after which sufficient glass-distilled water was added to make 5 ml. Control flasks were run that contained 0.15 ml of a solution containing 0.03 ml of 2N NaOH per 5 ml, and the addition of this weakly basic solution had no effect on the malic dehydrogenase values.

The data presented in Table II indicate that the estrogenic compounds are efficient inhibitors of the malic dehydrogenase activity of rat liver *in vitro*. The 8 compounds used are listed in the order of increasing inhibitory power: Androsterone sulfate 2% inhibition, disodium-3,4-diphenylhexane - p,p'-dioxyacetate, 6%, No. 020 (benzestrol) 22%, dienestrol 36%, diethylstilbestrol 44%, hexestrol 75%, No. 221B2 (benzestrol) 90%, and No. 103 (benzestrol) 93%.

To determine whether malic dehydrogenase itself was inhibited, or whether the inhibition was effected through inhibition of cytochrome oxidase, cytochrome c was replaced by brilliant cresyl blue. Preliminary results indicated that the cytochrome c of the malic dehydrogenase system can be completely substituted for by brilliant cresyl blue. On the basis of these data and before doing further work on the inhibition of this system by the estrogens, it was decided to determine the amount of the dye required for optimum activity of malic

⁴ Williamson, S., and Green, D. E., *J. Biol. Chem.*, 1940, **135**, 345.

[†] We are indebted to the Abbott Laboratories and to Dr. C. W. Sondern, White Laboratories, for the stilbestrol type estrogens, to Dr. A. H. Stuart, Scheffelin Company, for samples of the benzestrol compounds, and to Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Inc., for the sample of sodium androsterone sulphate.

TABLE II.
Malic Dehydrogenase Activity of Liver with Estrogenic and Androgenic Inhibitors *in Vitro*.

Q_{O_2}		Inhibition, %	Avg, %
Control	Inhibitor Final molarity 10^{-4} M		
	Diethylstilbestrol.		
55.9	22.5	60	44
79.7	46.9	42	
82.6	57.3	30	
	Hexestrol.		
71.8	21.3	70	75
75.3	26.6	81	
	Dienestrol.		
90.7	60.3	34	36
90.8	34.2	63	
64.0	42.7	33	
65.8	38.4	32	
79.2	47.4	40	
80.6	74.8	7	
80.6	48.2	40	
	Disodium 3,4-diphenylhexane-p,p'-dioxyacetate.		
64.0	61.4	4	6
65.8	59.7	9	
76.5	77.5	0	
85.4	76.2	11	
	No. 020.		
84.2	65.2	23	22
78.3	62.6	20	
	No. 103.		
84.2	4.8	94	93
78.3	6.1	92	
	No. 221B2.		
90.7	10.4	89	90
90.8	8.0	91	
	Na Androsterone SO_4 .		
79.2	75.0	6	2
80.6	80.9	0	
80.6	80.9	0	

dehydrogenase. From the data of Table III it can be seen that there is a range of optimum dye concentration, above and below which the oxygen uptake of 0.25 ml of liver homogenate begins to decrease. This optimum range is from 0.125 to 0.75 mg of brilliant cresyl blue per flask contents contained in a total volume of 3 ml. As a result 0.5 mg of the dye was used per flask in the following experiments.

Using an amount of brilliant cresyl blue which gives maximum activity (0.1 ml of a 0.5% solution) varying amounts of tissue (from 0.05 ml to 0.35 ml) were used in an effort to determine whether the oxygen uptake was proportional to the concentration of tissue. As can be seen from the data given in Table IV, the Q_{O_2} decreases fairly rapidly with increasing amounts of liver homogenate which shows that the oxygen uptake was not

directly proportional to the amount of tissue reacting. Furthermore, when each of the reagents was increased in turn to determine whether there was an insufficient amount of one of the constituents, a direct proportionality between oxygen uptake and the amount of tissue used was not obtained.

The inhibiting power of diethylstilbestrol on the malic dehydrogenase activity of liver was tested using cytochrome c alone, brilliant cresyl blue alone and a combination of this dye and cytochrome c. A final concentration of 1×10^{-4} M diethylstilbestrol was used in these *in vitro* studies. The data of Table V indicate that brilliant cresyl blue can adequately take the place of cytochrome c in the malic dehydrogenase system in that under the conditions used the oxygen uptake is as high in the presence of the dye as in the presence of cytochrome c. This is in contrast

TABLE III.
Malic Dehydrogenase Activity of Liver with Various Concentrations of Brilliant Cresyl Blue.

Brilliant cresyl blue, mg/flask	QO ₂					
	Run I	II	III	IV	V	Avg
—	3.9	0.0	4.7	6.2		4
.0125	25.6	—	—	—		26
.025	52.8	—	—	—		53
.0375	62.1	—	—	—		62
.05	64.4	—	—	—		64
.0625	76.0	61.3	66.7	—		68
.125	76.0	77.6	72.9	—		76
.25	72.9	77.6	71.4	77.6		75
.325	71.4	69.1	77.6	—		73
.5	77.6	77.6	76.0	77.6	74.5	77
.75	76.0	77.6	69.8	71.4	—	74
1.0	68.3	55.1	77.6	62.9	55.9	64
2.0	57.4	62.9	60.5	59.8		60
3.0	66.0	59.8	65.2	53.5		61
4.0	49.7	77.6	62.9	43.5		58

TABLE IV.
Malic Dehydrogenase Activity with Brilliant Cresyl Blue* and Varying Concentrations of Liver Homogenate.

QO ₂						
0.05 ml	0.1 ml	0.15 ml	0.2 ml	0.25 ml	0.3 ml	0.35 ml†
91.7	92.4	85.7	77.0	77.0	74.1	
—	74.2	74.6	—	71.5	66.4	57.5
106.7	90.6	108.1	82.0	66.4	—	
—	—	85.6	80.9	71.2	—	
—	88.9	74.2	85.0	71.0	—	
71.2	87.6	73.9	71.6	60.7	—	
71.2	68.2	68.4	58.5	57.3	60.9	
106.9	74.1	62.4	67.8	68.0	56.1	
Avg	89.5	82.3	74.7	67.9	64.4	57.5

* Concentration of brilliant cresyl blue was 0.5 mg per flask.

† Liver homogenate was 2.5%.

to the succinoxidase system in which the activity is decreased considerably when the dye is substituted for cytochrome c.^{5,1} The fact that this dye is autoxidizable in the presence of oxygen permits the malic dehydrogenase system to function without the action of cytochrome oxidase. Thus, if the estrogen inhibition is taking place by way of cytochrome oxidase, the addition of brilliant cresyl blue to the system should result in the malic dehydrogenase being essentially as active in the presence as in the absence of the inhibitor. The data of Table V show that this is the case. Diethylstilbestrol in a final concentration of 1×10^{-4} M is an effective in-

hibitor of the malic dehydrogenase system when cytochrome c is used, but when it is replaced with brilliant cresyl blue there is little inhibition, and there is even less when both cytochrome c and dye are present. These results support the concept that the estrogens inhibit the malic dehydrogenase system through action with the cytochrome oxidase of the system and not by direct action with the malic dehydrogenase.

Furthermore, only those compounds that have phenolic groups inhibit the malic dehydrogenase system, and when these groups are replaced by oxyacetate groups little, if any, inhibition occurs. Also sodium androsterone sulphate which contains an alcoholic in place of a phenolic group does not show inhibitory

⁵ Weil-Malherbe, H., *Biochem. J.*, 1937, **31**, 299.

TABLE V.
Inhibition by Diethylstilbestrol of Malic Dehydrogenase of Liver Using Cytochrome c and Brilliant Cresyl Blue.

Q _{O₂}					
Cytochrome c		Cresyl blue		Cytochrome c-Cresyl blue	
Control	Inhibitor*	Control	Inhibitor*	Control	Inhibitor*
84.3	34.4	82.2	62.9	—	—
62.9	3.5	69.0	61.6	—	—
70.0	13.0	73.9	71.0	77.5	71.3
75.3	27.2	78.7	75.6	71.3	68.9
71.2	5.9	67.9	59.8	64.0	61.9
71.2	7.1	69.2	58.3	64.0	60.4
74.8	11.8	75.1	63.0	70.1	67.8
Avg	72.8	14.7	73.7	64.6	69.4
					66.1

* Diethylstilbestrol, final concentration 1×10^{-4} M.

action. This does not seem unreasonable as cytochrome oxidase, through which the inhibition was shown to be effected, is known to catalyze the oxidation of certain phenolic compounds. It is suggested that there is an affinity, therefore, between the active centers of the enzyme and phenolic groups. Thus, it appears that the phenolic groups of the estrogenic compounds combine with the active centers and remain attached to the enzyme which prevents the latter from acting. This mechanism was shown previously to account for the inhibition of the succinoxidase system by estrogens.¹

Summary. The malic dehydrogenase activity of liver homogenate was shown to be proportional to the amount of tissue used.

It was found that the cytochrome c of the malic dehydrogenase system could be replaced by brilliant cresyl blue. The maximum activity of the dehydrogenase was obtained when the concentration of the dye ranged

from 0.125 mg to 0.75 mg per flask.

The inhibitory action of one androgenic and 7 estrogenic compounds was tested on liver malic dehydrogenase *in vitro*. Stilbestrol and benzestrol estrogens are effective inhibitors, but little inhibition occurs when the phenolic groups of the stilbestrol type compounds are replaced by oxyacetate groups as for example in disodium 3,4-diphenylhexane-p,p'-dioxyacetate. In addition sodium androsterone sulphate which contains an alcoholic in place of a phenolic group shows very little inhibitory action. It appears that the presence of phenolic groups are necessary for inhibition to take place.

It was concluded also from inhibitor studies with diethylstilbestrol, in which brilliant cresyl blue was substituted for cytochrome c, that diethylstilbestrol exerts its inhibitory action on cytochrome oxidase rather than malic dehydrogenase.

The Presence of Ferritin in the Duodenal Mucosa and Liver in Hemochromatosis.

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The etiology of hemochromatosis is unknown.¹ Recently, work on ferritin has indicated that this substance is a normal iron storage protein of the body. Ferritin, by virtue of its storage property also appears to function in the regulation of iron absorption from the gut. The opportunity was presented of examining whether a relationship might exist between factors governing ferritin formation and those which lead to huge deposits of iron in hemochromatosis.

Case history: Presbyterian Hospital unit number 861951. Admitted March 15, died May 6, 1947.

The patient was a housewife of 56, who had had diabetes mellitus and an enlarged liver for seven years. *B. coli* peritonitis, and bilateral oophorectomy in 1945. She developed congestive heart failure a year before admission, without valve lesions or hypertension. Bronzing of the skin was not noticed until it was called to her attention. During her stay in the hospital auricular fibrillation and ascites appeared. Her diet was difficult to control, as she was disoriented at times. Small doses of standard insulin were used. The laboratory findings were: RBC 4,400,000, Hgb. 13.0 g, WBC 11,900, differential normal. Serum alkaline phosphatase 10.1 Bodansky units, urea N 14 mg%, bilirubin, trace, cholesterol 213 mg%, albumin 3.2%, globulin 2.4%, cephalin flocculation +, prothrombin time 24 sec., blood sugar varied from 70 to 200 mg%, serum CO₂ content 55 vol.%. A biopsy from the left upper quadrant of the abdomen showed normal muscle, but hemo-

siderosis of the skin compatible with hemochromatosis. She died after 6 weeks, in congestive failure.

Autopsy number 15,292: Death 12:15 a.m., autopsy 2:00 a.m. Organs placed in ice-box, delivered to the Rockefeller Institute 9 a.m. The findings were typical of hemochromatosis, both in the gross and microscopically, with marked cirrhosis of the liver, and fibrosis of the myocardium. There was an old mural thrombus at the apex of the left ventricle, infarcts of liver, spleen and right kidney, and an embolus in the left common iliac artery. A terminal bronchopneumonia and other incidental findings were noted. The liver weighed 2,210 g, spleen 220 g, and heart 340 g. The pancreas showed moderate siderosis, the islets were not remarkable. The pituitary showed a large scarred area in the pars anterior.

Experimental. Ferritin was isolated from the liver and spleen by a procedure described previously.² The human ferritin precipitates from solution as brown spheroids, instead of well-defined crystals when CdSO₄ is added to a solution of ferritin. Further purification was attempted by dissolving the CdSO₄-precipitate in ammonium sulfate (4 g/100 cc). On increasing the ammonium sulfate concentration up to 10 g/100 cc, a brownish slimy material could be centrifuged away. The remaining deep brown solution precipitated out completely on dialysis against distilled water. This precipitate was dissolved by bringing it to pH 5 with acetate buffer and the properties of the resulting solution were examined.

Addition of CdSO₄ to an aliquot of the solution resulted in a precipitate of brown spheroids with no suggestion of crystalline shape, indicating that this material was still impure.

* The writers are indebted to Doctors Randolph West and H. P. Smith of the Departments of Medicine and Pathology at Columbia University for the clinical record and the pathological material studied.

¹ Sheldon, J. H., *Hemochromatosis*, Oxford University Press, London, 1935.

² Granick, S., *J. Biol. Chem.*, 1943, **149**, 157.

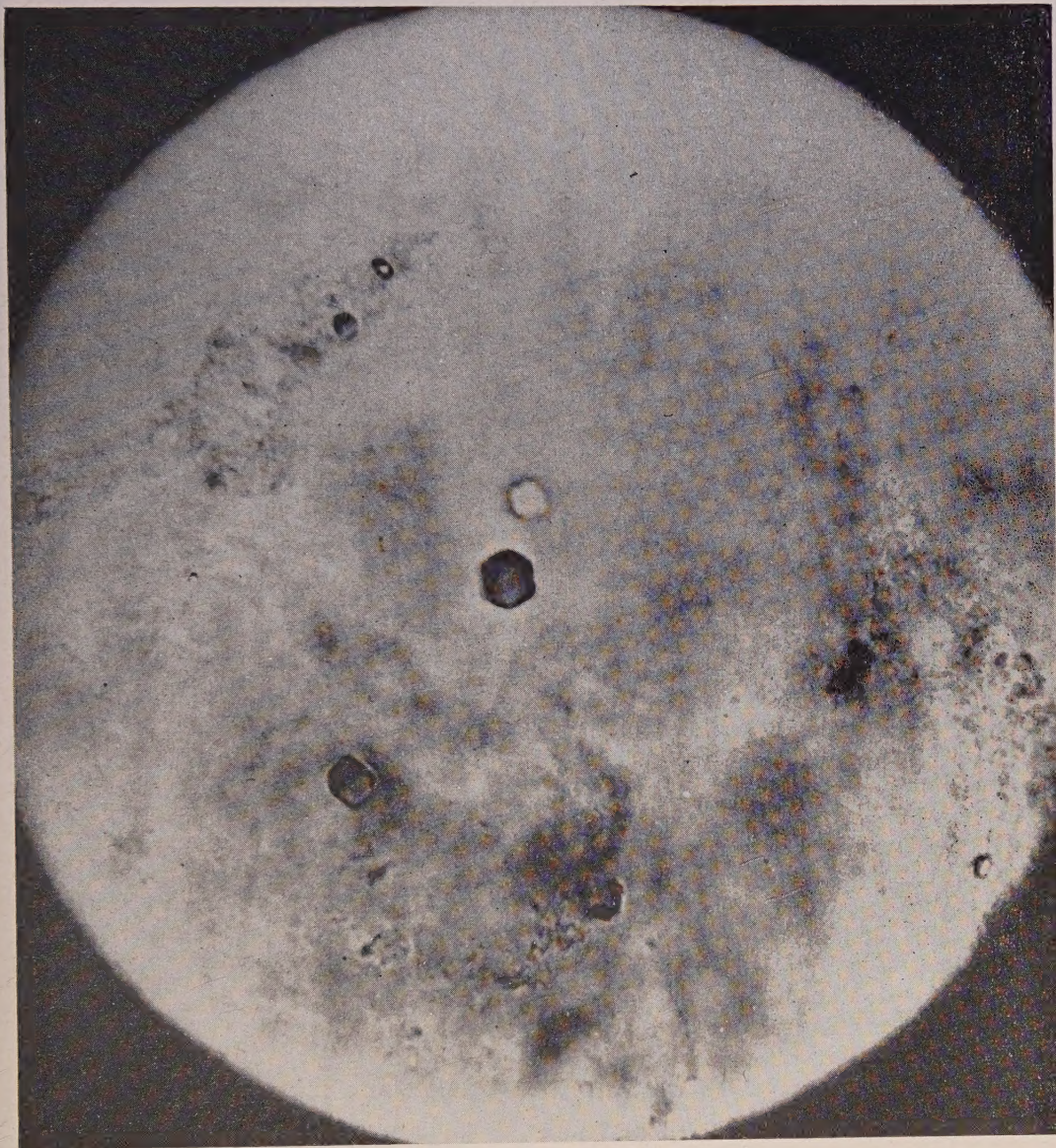


FIG. 1.

The solution contained 5.73 mg Fe/cc and 0.858 mg P/cc, or a ratio of 3.7 Fe : 1 P atom. The phosphorus appeared to belong to the iron hydroxide-phosphate micelles of ferritin since all of the phosphorus and iron could be dialyzed away on treating this ferritin with hydrosulfite at pH 4.6. This material was higher in P than was horse ferritin where the ratio is 8 or 9 Fe to 1 P atom. The ferritin contained 16.5% by dry weight of iron.

Magnetic measurements on this sample gave a value of 3.90 Bohr magnetons (B.M.) per g atom of Fe, the susceptibility being measured at 25°C. This value agrees within the limits of error with the value obtained for ferritin iron from horse and dog.³ A sample of normal human ferritin kept in the ice-box for 3 years gave a value of 3.94 B.M.

³ Michaelis, L., Coryell, C. D., and Granick, S., *J. Biol. Chem.*, 1943, **148**, 463.

Reprecipitation of the brown solution with CdSO_4 yielded about 1.2 cc of packed globules per 100 g of fresh liver. This is the highest content of ferritin yet obtained from human livers.² Ferritin was also isolated from the spleen and had properties of solubility and precipitability with CdSO_4 similar to those of the liver material. However, only about 0.11 cc of packed reprecipitated ferritin globules per 100 g fresh spleen were isolated.

A homogenate, prepared from a piece of liver by grinding in a Waring blender and further comminuting in a TenBroeck grinder was subjected to magnetic measurements. It had a value of 4.0 B.M. per g Fe atom. The iron in this homogenate consisted for the most part of ferritin and of hemosiderin granules, with a relatively negligible contribution from the iron porphyrin pigments. It is interesting that this value is not essentially different from the value of ferritin itself, probably indicating that the hemosiderin granules in the liver contain iron in the same structural configuration as in ferritin. A somewhat lower value for hemosiderin granules was obtained from horse spleen (B.M.=3.4).³

The duodenal mucosa was tested for ferritin by adding CdSO_4 to scrapings of the mucosa on a glass slide and examining it after 24 hours. Brown crystals of ferritin were observed in this material (Fig. 1). The crystals were better defined, with sharper outlines, than any human ferritin crystals seen previously. The presence of the crystals is evidence for the presence of ferritin in the duodenal mucosa. Normal human mucosa has

not yet been examined for the presence of ferritin.

Discussion. The fact that ferritin can be found in the duodenal mucosa, spleen, and especially abundantly in the liver, indicates that hemochromatosis is not brought about by a failure to produce the specific protein apoferritin. The magnetic measurements on the ferritin of the liver also indicate that the structural configuration of the iron hydroxide-phosphate of the ferritin is normal, although its phosphorus content appears to be somewhat high.

The idea has often been suggested that the mechanism governing the intake of iron through the intestinal mucosa might be damaged or non-functional in hemochromatosis. If ferritin is assumed to play a role in the regulation of iron absorption⁴ then the presence of ferritin in the duodenal mucosa does not support the idea that the regulatory mechanism of iron-absorption is directly affected.

In general, the normal blood picture in hemochromatosis and also the high level of iron in the serum suggest that the transport of iron is adequate.

The extremely high ferritin content of the liver and the normal magnetic values for ferritin iron indicate that the mechanism for iron storage is functioning at capacity.

Conclusion. The cause of hemochromatosis does not appear to be a defect in the formation of ferritin in the duodenal mucosa or the liver.

⁴ Granick, S., *J. Biol. Chem.*, 1946, **164**, 737.

Sparing Action of Protein on Pantothenic Acid Requirement of Rat. III. Fibrin as the Protein Component.*

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The sparing action of protein on the pantothenic acid requirement of the rat was first demonstrated by Nelson and Evans¹ with purified diets, deficient in pantothenic acid, containing 64% instead of 24% casein. When intermediate levels (24%, 34%, 44%, 54%, and 64%) of casein were used, the sparing action as judged by growth, survival and urinary excretion of pantothenic acid of deficient rats was proportional to the casein level.² The presence of small amounts of "combined" pantothenic acid in the casein used (and in all types of purified casein tested) prompted a study of the effects of 24% casein diets supplemented with the amounts of pantothenic acid occurring in the casein of the 44% and 64% casein diets. The results showed that the pantothenic acid content of the purified casein used, while exercising an effect, could not fully account for the protective action of the high casein diets.²

However, it was felt highly desirable that a protein free from or considerably lower in pantothenic acid than casein be used in the further investigation of this problem. Preliminary assays indicated that beef blood fibrin, purified by washing with water or with dilute acid, was extremely low in pantothenic acid. The present communication reports the growth and survival of pantothenic acid deficient rats maintained on diets containing two levels of purified fibrin, 24% and 48%, and

on the corresponding casein-containing diets.

Experimental Procedure. Dried crude beef blood fibrin (Armour and Company) was purified by washing with water for one week.[†] One kg of crude fibrin was placed in a 20-gallon crock and 15 gallons of water (either tap or distilled) added to it. The mixture was stirred thoroughly and allowed to settle. The supernatant liquid was siphoned off 2-4 times daily and fresh water added. Ten cc of chloroform were used with each change of water as a preservative. After 5-7 days of washing, the supernatant was drained off and the fibrin filtered through a large Buchner funnel. Suction was maintained for several hours or overnight if necessary to dry out the fibrin cake. It was then broken up by sieving and dried for 24 hours in a low temperature oven (50°C). After grinding, the fibrin is ready for use in the diets.

Microbiological assays for pantothenic acid were carried out by the method of Hoag, Sarett, and Cheldelin³ with crystalline B vitamins substituted for the rice bran extract and autolyzed yeast in the assay medium. Enzymatic digestion with clarase or mylase P for 24 hours at 37°C⁴ followed by the 24-hour assay procedure was used for the purified fibrin and casein. The water-washed fibrin used in the diets averaged 0.5 μ g pantothenic acid per g whereas the alcohol-extracted casein contained 1.5-1.8 μ g per g. There was considerable variation in the pantothenic acid content of different lots of fibrin after washing and additional purification was sometimes

* Aided by grants from the Roche Anniversary Foundation, the Board of Research and the Department of Agriculture of the University of California and the Rockefeller Foundation, New York City. Crystalline B vitamins and alpha-tocopherol were generously contributed by Merck and Company, Inc., Rahway, N.J.

¹ Nelson, M. M., and Evans, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 319.

² Nelson, M. M., van Nieuhuys, F., and Evans, H. M., *J. Nutrition*, 1947, **34**, 189.

[†] The procedure for washing the fibrin was that used by D. H. Copp (Department of Physiology, University of California).

³ Hoag, E. H., Sarett, H. P., and Cheldelin, V. H., *J. Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 60.

⁴ Ives, M., and Strong, F. M., *Arch. Biochem.*, 1946, **9**, 251.

necessary. Other methods of purification are being investigated in order to prepare fibrin completely free of pantothenic acid for balance studies.

The basal diet contained 24% alcohol-extracted casein or water-washed fibrin, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco) and 4% salts No. 4.⁵ The proportion of casein or of fibrin in the higher protein diets was increased to 48% at the expense of the sucrose. All diets contained the following crystalline vitamins per kg diet: 5 mg 2-methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 400 mg inositol, and one g choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 650 mg corn oil (Mazola), 800 U.S.P. units vitamin A, 115 Chick Units vitamin D, and 6 mg alpha-tocopherol. The basal diet containing 24% casein is the high-carbohydrate diet used in previous studies^{1,2} with the addition of 2-methyl-1,4-naphthoquinone to the vitamin mixture. The previous weekly allowance of the fat-soluble vitamins has been doubled.

In the first series of animals, 20 litters of male rats (Long-Evans strain) were divided into 4 equivalent groups and placed on the pantothenic acid deficient diets containing 24% or 48% casein or fibrin at weaning. An additional series of 10 litters of male rats were divided into equivalent groups and placed on the 2 fibrin diets at weaning. All animals were maintained in individual cages provided with screens to prevent coprophagy.

Results and Discussion. The growth of littermates (20 rats per group) maintained on 24% and 48% casein or fibrin diets deficient in pantothenic acid is shown in Fig. 1, while Table I presents the data on growth and survival for the combined series of animals. The sparing effect on growth and survival of doubling the casein level (24% to 48%) is evident although the effect is not as great as it would be in a longer experimental period or with higher casein levels.² The substitution of purified fibrin in the 24% casein diet resulted in

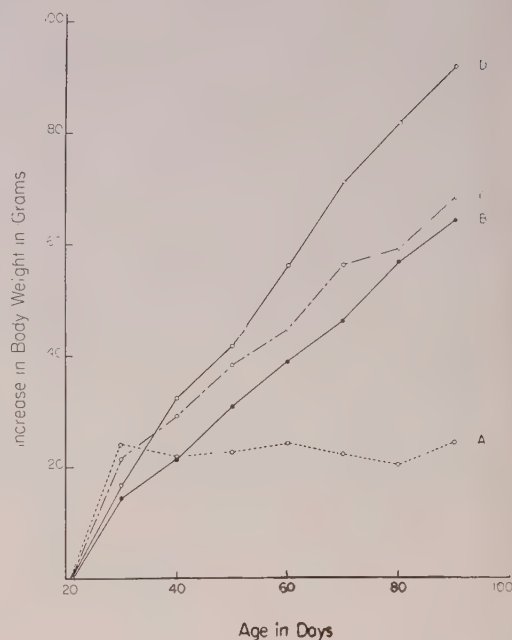


FIG. 1.

Growth of pantothenic acid deficient rats maintained on purified diets varying in casein or fibrin level.

- A. 24% fibrin diet.
- B. 48% " " "
- C. 24% casein " "
- D. 48% " " "

a marked accentuation of the deficiency, shown in the greatly decreased growth and survival in comparison to all other diets. Despite the severity of the deficiency, doubling the fibrin level (48% fibrin diet) resulted in significantly increased growth and survival. In fact, the differences in growth and survival were greater than those between the 24% and 48% casein diets during the short experimental period.

The accentuation of the deficiency observed on the 24% fibrin diet was not due to the lack of any essential factors in the purified fibrin. Control rats receiving the 24% fibrin diet supplemented with calcium pantothenate grew slightly faster than littermates receiving the supplemented 24% casein diet. This is in agreement with the findings of Risser⁶ that fibrin, both crude and pharmaceutical grades, was somewhat higher in biological value than casein. The decreased pantothenic acid con-

⁵ Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 460.

⁶ Risser, W. C., *J. Nutrition*, 1946, **32**, 485.

TABLE I.
Effect of Casein and Fibrin Levels on Growth and Survival of Pantothenic Acid Deficient Rats.

Dietary protein	No. of rats	Survival			Avg body wt Day 60 g	Survival		Avg body wt Day 90 g
		Day 30 %	Day 45 %	Day 60 %		Day 75 %	Day 90 %	
24% fibrin	30	83	60	23	72	3	3	73
48% " "	30	97	77	57	86	43	33	111
24% casein	20	100	80	60	93	50	40	117
48% " "	20	100	95	85	105	55	45	132

tent of the washed fibrin is the obvious factor responsible for the accentuation, although other possibilities such as the presence of accentuating factors, decreased intestinal synthesis, or the lack of biotin and folic acid^{7,8} cannot be eliminated without investigation. Sarma, Snell and Elvehjem⁹ have reported an accentuation of pyridoxine deficiency when fibrin-containing diets were used and believed that the accentuation was greater than could be accounted for by the decreased pyridoxine content of the fibrin diets.

Fibrin and casein are similar in amino acid content¹⁰ but are markedly different in regard to phosphorus content. Fibrin is extremely low in phosphorus and is frequently used for studies of phosphorus deficiency, while casein a conjugated phosphoprotein, contains somewhat less than 1% phosphorus. Doubling the proportion of fibrin in the diet does not significantly affect the phosphorus content whereas doubling the proportion of casein markedly increases the phosphorus concentration of such diets. The phosphorus content of the diets used (calculated from the phosphorus

analyses of purified fibrin and casein[†] and the composition of the salt mixture) are as follows: 24% fibrin diet—0.6%; 48% fibrin diet—0.6%; 24% casein diet—0.7%; and 48% casein diet—0.9%. The fact that an increased phosphorus content is unnecessary for the sparing action of high fibrin diets indicates that phosphorus can be eliminated as a possible factor in the sparing action of protein on the pantothenic acid requirement of the rat.

Summary. The sparing action of protein on the pantothenic acid requirement of the rat has been confirmed by using a protein considerably lower in pantothenic acid, washed beef blood fibrin, instead of casein as the protein component of the diet. Despite the accentuation of the pantothenic acid deficiency produced by substituting purified fibrin for casein at the 24% level, doubling the fibrin level resulted in significantly better growth and survival. The differences in growth and survival between the two protein levels in the fibrin-containing diets were more pronounced than those observed for the casein-containing diets. The protective effects of the high fibrin diet indicated also that phosphorus can be eliminated as a possible factor concerned in the sparing action.

[†] The phosphorus analyses were carried out by Miss Genevieve Beach through the courtesy of D. H. Copp. The water-washed fibrin contained 0.045% and the alcohol-extracted casein 0.61% phosphorus.

⁷ Wright, L. D., and Welch, A. D., *Science*, 1943, **97**, 426.

⁸ Wright, L. D., and Welch, A. D., *J. Nutrition*, 1944, **27**, 55.

⁹ Sarma, P., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **165**, 55.

¹⁰ Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods*, 1945, Chapter X, p. 300, Charles C. Thomas, Springfield, Ill.

The Age Factor in the Castration of Male Rabbit Fetuses.

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In previously reported experiments on intra-uterine surgical castration of rabbits, it was ascertained that the fetal gonads play some part in somatic sexual differentiation. In controls, the first somatic sexual differences appear on the 20th day;¹ and prostate buds appear at 21 days. At 27-28 days, genital organogenesis is practically completed; all control and experimental fetuses were dissected and preserved at that age. The following observations were published recently: (1) males gonadectomized at 23 days (4 cases) acquired nearly all male characters, but the deferent ducts and ampullae were partly involuted;^{2,3} (2) in males castrated at 22 days (2 cases) the wolffian ducts had disappeared, but vestiges of mullerian ducts were present; the prostate was reduced and the external genitalia were abnormal.⁴ (3) In a group of males castrated at 21 days, 3 out of 8 retained some wolffian vestiges; the other 5 had none; the mullerian ducts persisted and had differentiated into mullerian vagina, uterine horns, and sometimes, oviducts. In microscopic sections, the external genitalia presented a feminine aspect. Only 2 rudimentary prostatic buds had developed; they correspond to the cranial prostate, which is first to appear in normal development. (4) There were great similarities between fetuses of both sexes castrated at this age, but only the males had prostatic buds.⁵

It was the aim of the now to be presented new series of experiments to determine: (1) whether male fetuses may acquire and retain normal male characters, if castrated after the

23rd day, and (2) whether their prostate development may be suppressed completely, if castrated before the 21st. The following results were obtained.

Two males, gonadectomized at 24 days, continued to develop according to the male type, retaining their deferent ducts and ampullae. The size of the prostate is somewhat below normal.

In fetuses operated before the age of 21 days, the following conditions were observed: 3 males castrated at 20 days developed and retained female genital tracts, but also had 2 prostatic buds. In 2 males gonadectomized at 18 days 19 hours, and 19 days 5 hours, *i.e.*, before the initiation of somatic sexual differentiation, prostatic development was entirely suppressed. Another male, castrated at the same age, and treated with testosterone propionate, exhibited a large prostate.

Thus, in order to suppress completely the formation of prostates, it is necessary to castrate the embryos before the development of their primordia. After primordia have reached a certain stage of development, especially from the 23rd day on, they continue growing and branching, in spite of the absence of the testes. However, they are somewhat reduced in size; this point should be checked by a quantitative study like that devoted to the rat by Wells.⁶ One may wonder whether Moore's opossums were already too old for prostate development to be suppressed when they were gonadectomized.⁷

Three groups of facts suggest that the testis exerts its influence by means of actual secretions: (1) in males unilaterally castrated at 22 or 23 days, the wolffian ducts are fully

¹ Jost, A., *C. R. Soc. Biol.*, 1943, **137**, 586.

² Jost, A., *C. R. Soc. Biol.*, 1946, **140**, 774.

³ Jost, A., *C. R. Soc. Biol.*, 1946, **140**, 938.

⁴ Jost, A., *C. R. Soc. Biol.*, 1947, **141**, 126.

⁵ Jost, A., *C. R. Soc. Biol.*, 1947, **141**, 135.

⁶ Wells, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 417.

⁷ Moore, C. R., *J. Exp. Zool.*, 1943, **94**, 415.

maintained;^{3,4} (2) androgens have a reparative influence;⁸ (3) the results of one especially favorable case of testis graft in a female showed localized inhibition of the oviduct, more extensive persistence of the wolffian duct.

Some new observations contribute complementary data on the manner of action of the testicular secretion. In 3 males unilaterally castrated between the ages of 19 days 6 hours and 20 days 21 hours, the prostate and the external genitalia are well developed, but the single testis acts less effectively on the primordia of the opposite side. In a fetus with only a right testis, of somewhat under normal size, the vas deferens is present only on the right side, while on the left, a uterine horn persists. It follows that after early unilateral gonadectomy the action of the fetal testis is relatively localized. A single testis or a fragment of a testis (as appears in the case of an incomplete castration) does not seem to undergo compensatory secretory hypertrophy;

⁸ Jost, A., *C. R. Soc. Biol.*, 1947, **141**, 275.

with regard to this point, it should be remembered that the hypophysis is not indispensable for embryonic sexual differentiation.^{9,10,11} Lastly, some of our observations suggest that more testicular secretion is required to completely inhibit the mullerian ducts, than to maintain the wolffian ducts.

Conclusion. In the male rabbit, early fetal castration (before the 21st day) prevents the formation of male secondary sex characters such as the prostate. At a later stage (24 days) established primordia continue their development even after ablation of the testes; the resulting structures are usually subnormal. The effects induced by testicular secretions are sometimes regionally restricted. Earlier observations on the differentiation and persistence of female genital ducts after early orchidectomy are confirmed and extended.

⁹ Ancel, P., *Les Hormones Sexuelles*, Colloque Singer Polignac, Paris, 1937, Hermann.

¹⁰ Wolff, E., and Stoll, R., *C. R. Soc. Biol.*, 1937, **126**, 1215.

¹¹ Fugo, N., *J. Exp. Zool.*, 1940, **85**, 271.

16072

The Lymphoid Tissue and Antibody Formation.

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Several early investigators in immunology considered the possibility that the spleen was the main site of antibody formation, but conclusive evidence for this was not found. Hektoen,¹ using the well-known fact that X-ray produces a destructive effect on all lymphoid tissue, demonstrated that antibody formation is restrained to a marked degree in X-rayed animals. He concluded from this observation that the spleen, lymphoid tissue and bone marrow were the principal sites of immune bodies. Murphy and Sturm² confirmed Hek-

toen's findings but considered that they had eliminated the bone marrow as of first importance in the process. This conclusion was based on the fact that depletion of the lymphoid tissue was effected by repeated, small doses of X-ray of low penetration which produced no detectable damage to the bone marrow or other tissues. Yet animals treated in this manner were found deficient in the production of antibodies. In addition, these investigators demonstrated that rabbits, after stimulation of the lymphoid system by dry heat treatment, had an enhanced ability to produce immune substances. Hussey³ in this

¹ Hektoen, L., *J. Infect. Dis.*, 1915, **17**, 415; 1918, **22**, 28; 1920, **27**, 23.

² Murphy, J. B., and Sturm, E., *J. Exp. Med.*, 1925, **41**, 245.

³ Hussey, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1921, **19**, 22.

laboratory found that if the lymphoid system of guinea pigs is kept in a depleted state by X-ray following a sensitizing injection of horse serum, the animals show no anaphylactic manifestations on receiving a second injection of serum. In such animals the antigen continues free in the circulation as long as the X-ray is continued. McMaster and Hudack⁴ have clearly demonstrated that antibodies may be formed in lymph nodes but their experiments did not eliminate the possibility that cells other than lymphocytes participated in the reaction. Ehrich and Harris⁵ have extended these observations and on the basis that the tissue response accompanying the formation of antibodies in the nodes is predominately a lymphoid one, they consider these cells as the important factor in the development of immune substances. Harris, Grimm, Martens and Ehrich,⁶ in a study of the antibodies in the lymph, found when the lymphocytes are separated from the lymph plasma that the titer of the cell extract was substantially and consistently higher than that of the lymph plasma. This is taken to indicate that the lymphocytes either produce antibodies or take them up from lymph plasma. As no evidence for the latter was found, they believe that the lymphoid cells are the responsible agents. Dougherty, Chase and White⁷ noted that the antibody content of the blood is increased following the administration of adrenal cortical extract, a fact which they attributed to the destructive action of the hormones on the lymphocyte with the release of the antibodies supposedly formed in the cytoplasm of these cells. Philips, Hopkins and Freeman⁸ found no such increase when the lymphoid tissue is destroyed by nitrogen mustard and such treatment may result in an actual depres-

sion of the production of antibodies.

In the present investigation an estimate has been made of the antibody formation in animals with extreme hypertrophy of the lymphoid system which follows the removal of the adrenal glands. The release of the antibodies from the lymphocyte under these conditions would be due to the normal shedding of the cytoplasm as the cell matures. In the absence of the adrenals there can be no question of the cortical hormones playing any part in the reaction.

Circulating Lymphocytes in Adrenalectomized Rabbits. The circulating lymphocytes show a definite but not marked rise following removal of the adrenals. This increase is in the average range of 4000 cells per mm³, which is considerably less than that observed for mice and rats. However, if adrenalectomized rabbits are given an antigen, the lymphocyte count shows a marked increase up to an average of over 14,000 cells while normal rabbits under the same condition have only a slight increase.

Antibody Formation in Adrenalectomized Rabbits. Experiment 1. The adrenals were removed from 2 rabbits and immediately afterwards these animals and normal controls were given 10 cc of horse serum intraperitoneally. Ten days later, all were bled and the sera tested for precipitins. The sera from the adrenalectomized rabbits gave massive precipitates in the low dilutions, and definite positive reactions in dilutions of 1 to 640 in one serum and in dilutions of 1 to 2560 in the other. The serum from the control gave far less precipitate in low dilutions and there were no precipitates in dilutions higher than 1 to 320.

Exp. 2. The adrenals were removed from 7 rabbits and between 2 and 3 weeks later these with four controls were given five intravenous injections of horse serum, consisting of 1 cc, at 2-day intervals. All of the rabbits were bled 10 days following the last injection and the sera tested for precipitins. The results are given in Table I. It will be noted that all of the sera from adrenalectomized rabbits gave definite precipitates in dilutions up to 1:2560 and 4 were positive in 1:5120. Only

⁴ McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1935, **61**, 783.

⁵ Ehrich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, **76**, 335; *Science*, 1945, **101**, 28.

⁶ Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.*, 1945, **81**, 73.

⁷ Dougherty, T. F., Chase, J. H., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 28; 1945, **58**, 135.

⁸ Philips, F. S., Hopkins, F. H., and Freeman, M. L. H., *J. Immunol.*, 1947, **55**, 289.

TABLE I.
Immunized with 5 Injections of 1 cc Horse Serum Intravenously at 2-Day Intervals, 2-3 Weeks After Adrenalectomy

Dilutions	1:1	1:25	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
1	++		+	+	+	+	+	+	+	+	+	+	+
2	++		+	+	+	+	+	+	+	+	+	+	+
3	++		+	+	+	+	+	+	+	+	+	+	+
4	++		+	+	+	+	+	+	+	+	+	+	+
5	++		+	+	+	+	+	+	+	+	+	+	+
6	++		+	+	+	+	+	+	+	+	+	+	+
7	++		+	+	+	+	+	+	+	+	+	+	+

Dilutions	1:1	1:25	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
8	++		+	+	+	+	+	+	+	+	+	+	+
9	++		+	+	+	+	+	+	+	+	+	+	+
10	++		+	+	+	+	+	+	+	+	+	+	+
11	++		+	+	+	+	+	+	+	+	+	+	+
12	++		+	+	+	+	+	+	+	+	+	+	+

one control serum was positive in dilution of 1:2560 and in the others there was no reaction above 1:320. Far more striking than the difference between the two groups shown by dilution, is the difference in the volume of the precipitates in the lower dilution (Fig. 1).

Exp. 3. This experiment was carried out to compare the development of agglutinating antibodies in adrenalectomized and intact rabbits. A 24-hour slant culture of *Salmonella enteritidis* (mouse typhoid) was washed off with saline and the suspension heated on 3 consecutive days for 15 minutes, at 56°C. In preliminary tests it was found that while intact rabbits would withstand the intravenous inoculation of 1/10 of a culture, the adrenalectomized ones were killed by 1/1000 given intravenously and by 1/100 of a culture inoculated subcutaneously. The following method of immunization proved successful. Two adrenalectomized rabbits and two controls were each given 5 subcutaneous inoculations at 4-day intervals, starting with 1/300 of a killed culture and gradually increasing the amount up to 1/10 for the last inoculation. The animals were bled 10 days after the last injection and the sera tested for agglutinins against the organisms. The sera from the intact rabbits agglutinated up to dilutions of 1 to 40 and 1 to 80, respectively. The serum from the first adrenalectomized rabbit reacted at dilutions of 1 to 640 and the other was definite at 1 to 1280, the highest dilution used.

Effect of Adrenal Cortical Hormones on Antibody Formation in Adrenalectomized Rabbits. The marked over-development of the lymphoid system which follows removal of the adrenals may be prevented by the administration of adrenal cortical hormones. In the following experiment, the effect of suppressing the lymphoid development by the hormone treatment was tested on the antibody formation.

Two adrenalectomized rabbits were given 23 daily injections of 1 cc each of adrenal cortex hormones in oil, which was equivalent to 40 rat units or 2 mg of 14-oxy-11-dehydrocorticosterone (Upjohn Co.). This covered the period following the operation and extended through the period of immunization

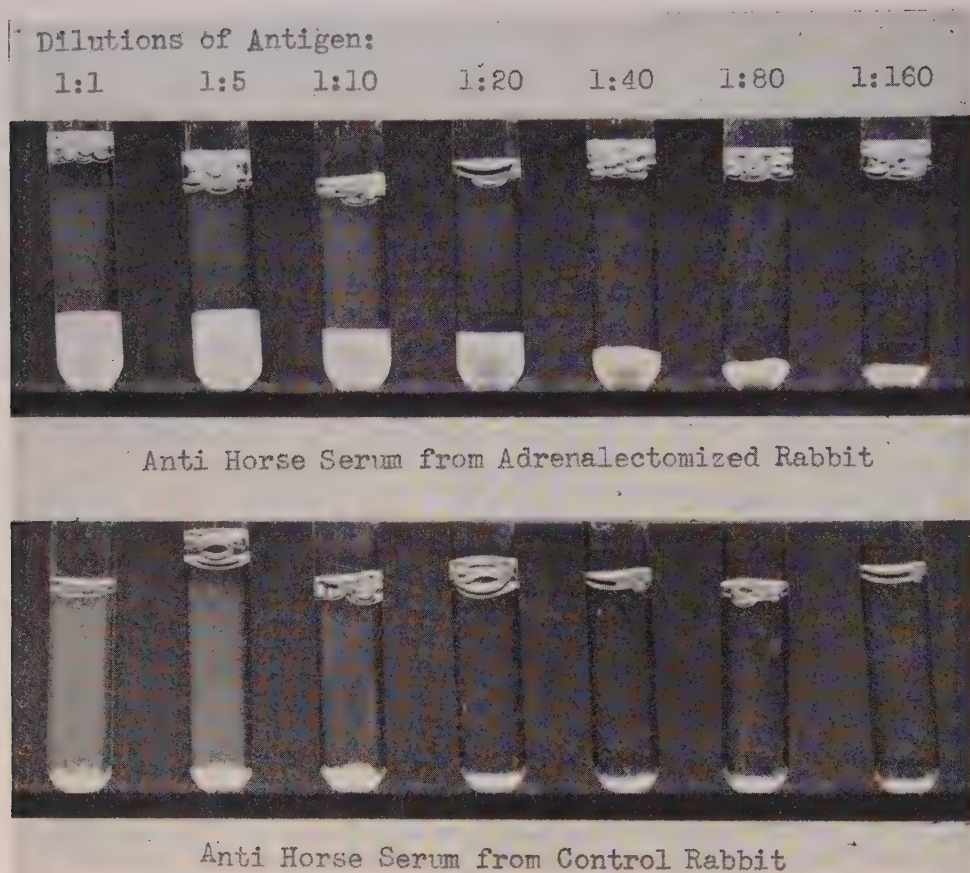


FIG. 1.

with horse serum. A third rabbit was given daily doses of one cc of Eschatin (Parke Davis and Co.). Each cc of this preparation is equivalent to 50 dog units. These animals with 2 untreated adrenalectomized rabbits and 2 intact controls were immunized with horse serum as in the previous experiment. The test of the sera showed no material difference in precipitin formation in hormone treated and untreated adrenalectomized rabbits. The intact controls gave far less response as in the preceding test.

Discussion. Ehrich and Harris^{5,6} in a series of papers have adequately discussed the evidence that the lymphocyte is a factor in antibody formation. The experiments reported above are essentially a confirmation of our earlier observation,² that animals with hypertrophied lymphoid systems have an enhanced ability to produce antibodies. In the earlier

tests dry heat was used as the stimulus; in the present study advantage was taken of the well-known fact that extensive overdevelopment of the entire lymphoid tissue follows removal of the adrenals. If the immune substances are present in the cytoplasm of the lymphocyte and there is evidence for this belief, the release into the circulation is probably due to shedding, which is known to occur as the lymphocyte matures. One of our tests confirms the observation of Dougherty, Chase and White⁷ that the release of antibodies may be definitely augmented by adrenal cortical hormones. The present experiments demonstrate that the hormone is not essential for this release and therefore, strictly speaking, antibody production is at least not entirely under the control of the adrenals.

Summary. Adrenalectomized rabbits with hypertrophy of the lymphoid tissues produce

antibodies in amounts far in excess of that produced by intact animals. This is evident not only by definite reactions in higher dilution but by the more massive precipitate in low dilutions. The prevention of the lymphoid hypertrophy by administration of adrenal

cortical hormones does not reduce the amount of antibodies in the sera. This is probably due to the disruptive action of the hormone on the lymphocyte with a more rapid release of the immune globulin than would normally take place.

16073

Prevention of Experimental Arteritis in Dogs by Vitamin E.*

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In a recent publication¹ data were presented to show that the combination of a specified high fat diet and experimentally induced renal insufficiency in dogs is regularly followed by predictable lesions in the arterial system. The lesions have been found in elastic arteries, in muscular arteries, and in arterioles in practically every organ and tissue of the body with the exception of the kidney and liver. Essentially the lesion is an acute necrotizing arteritis whose closest human counterparts are periarteritis nodosa and rheumatic arteritis.

When it was found that the dietary factor was lipid in nature, the question naturally arose as to the effect of vitamin E—the so-called “fat anti-oxidant” vitamin—on these experimental arterial lesions.

Methods. The methods have been detailed in previous publications.¹ Briefly these consist of feeding selected table scrap to which is added each day 3.0 cc/kg of a commercial grade of cod liver oil. After this diet has been consumed for 8 weeks or longer, the kidneys are severely damaged by any one of several methods (heavy metal injury, bilateral nephrectomy, *Leptospira Canicola*—usually

uranium nitrate, 10.0 mg/kg injected subcutaneously) and the arterial system is examined both grossly and histologically when the dogs die or are sacrificed days to weeks later.

In the first series of 4 dogs vitamin E—one capsule containing 20 mg of mixed natural tocopherols—was given by mouth between 8 and 10 o'clock in the morning and the dogs were fed the diet of table scrap and cod liver oil around 4 o'clock in the afternoon. Following kidney damage the dogs usually quit eating on the fourth to seventh day. Administration of vitamin E capsules was continued until the dog died.

In the second series of 4 dogs vitamin E (in the same form as above) was not started until *after* the kidneys were damaged. The dogs were fed table scrap and cod liver oil for 8 weeks or longer, then a lethal dose of uranium nitrate was injected and a single capsule of 20 mg mixed natural tocopherols per day by mouth was started 0, 24, 48, and 72 hours after the heavy metal injury. Vitamin E therapy was continued until the dogs died in uremia.

In these 8 dogs the single capsule of 20 mg of mixed natural tocopherols amounted to 2.5 to 4.0 mg/kg/day.

Experimental Observations. The significant data on the 4 dogs fed vitamin E concomitantly with the specified high fat diet for 8 weeks or longer before kidney damage was

* This work was aided by a grant from The John and Mary R. Markle Foundation. The mixed natural tocopherols and helpful suggestions were furnished by Distillation Products, Inc.

¹ Holman, R. L., and Swanton, M. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, **63**, 87.

TABLE I.
Effect of Vitamin E on Incidence of "Experimental" Arterial Lesions.

Group	Dog No.	Body wt at time of renal injury, kg	Period on high fat diet before renal injury, weeks	Day following renal injury on which Vit. E was started	Survival interval following renal injury, days	Arterial* lesions
A Vit. E—fed concomitantly with high fat diet	45-84	5.8	8	—56	10	—
	45-85	5.9	9	—63	11	—
	45-86	7.5	10	—70	12	—
	45-87	9.4	12	—84	13	—
B Vit. E—Started after renal injury.	46-8	19.0	14	0	10	—
	46-9	13.7	14	1	8	—
	46-13	13.0	14	2	30	—
	46-12	13.7	14	3	20	+

* The grading of the lesions here is the same as that employed in previous publications, namely,
+ = gross lesion greater than 1 cm in maximum dimension;
++ = gross lesion less than 1 cm in maximum dimension;
+++ = lesion discovered in routine histological section;
— = negative.

induced are given in Table I, section A; and section B of this table gives the same data for the 4 dogs in which vitamin E was started 0, 1, 2, and 3 days *after* renal insufficiency was produced. There is a striking difference in the incidence of arterial lesions in these two groups as compared with similar experiments in which vitamin E was not administered (Table II).

Discussion. Since the pathogenesis of the arterial lesions related to high fat diet and renal insufficiency is not understood, there is no ready explanation for the protective action of vitamin E. The problem might be approached indirectly. If we assume that vitamin E exerts its usual "anti-oxidant" action, we can postulate pathogenesis as follows: During the 8 weeks or more of dietary feeding, one or more of the "toxic" substances (fatty acids are under suspicion) contained in the diet saturates the tissues with the excess spilling over in the urine. When this safety valve is destroyed, the excess piles up to "explosive" levels and the arterial lesions ensue.

If our present theory that the toxic substance is fatty acid proves correct, a corollary of it would be that the fatty acid damages collagen, for the first detectable anatomical change is edema, swelling, fragmentation and necrosis of collagen. The subsequent fibrin, "fibrinoid" and intense leukocytic reactions that are seen in the typical arterial lesion presumably represent the response of the body to the necrotic collagen. Why the assumed fatty acid selects collagen in certain sites of the body for this activity—also whether it acts directly on the collagen in these sites or does so by inactivating vitamin C (which it has been shown to do in aqueous solutions *in vitro*²) are not known. Attempts thus far to demonstrate any variations in ketone bodies in the urine or any consistent variations in the blood plasma lipids at different stages in the experimental procedures have not yielded definite confirmatory evidence for the above hypothesis.

² Ströhecker, R., and Buchholz, E., *Chem. Abstr.*, 1943, **37**, 5763.

TABLE II.
Effect of Vitamin E on Incidence of "Experimental" Arterial Lesions.

Diet	Weeks*	No. with lesions	
		No. in group	% positive
Table scrap + C.L.O.†	8-15	22/25	88.0
Table scrap + C.L.O. + Vitamin E	8-14	1/8	12.5

* Weeks of dietary feeding before production of renal insufficiency.

† C.L.O. = Cod Liver Oil.

Summary. 1. Twenty-two of 25 dogs fed a kennel diet of selected table scraps to which was added each day for 8 weeks or longer 3.0 cc/kg of cod liver oil developed typical arterial lesions when they died in uremia 7 to 35 days after the experimental production of renal insufficiency.

2. When vitamin E (mixed natural tocopherols, 2.5 to 4.0 mg/kg/day by mouth)

was administered along with the diet, none of 4 dogs subjected to the same experimental procedures developed any arterial lesions.

3. Vitamin E was equally effective in preventing or retarding the arterial lesions when its administration by mouth was started 0, 1, and 2 days after renal insufficiency was induced.

16074

Cataract Development in Animals with Delayed Supplementation of Tryptophane.

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In several recent papers¹⁻⁴ demonstrating the production of lesions in the eyes of experimental animals on inadequate diets, corneal vascularization was encountered more frequently and as a more general response to deficiencies than cataract formation. The latter lesion was observed only rarely, chiefly in rats fed on tryptophane or histidine deficient diets. The development of cataract might be a result of disturbed protein synthesis due to the absence of the essential amino acids; however, since cataract did not

develop in animals on diets deficient in other essential amino acids such as lysine, leucine, threonine, valine, isoleucine, arginine, etc., the possibility of a specific role of tryptophane and histidine in the metabolism of the lens must be considered. It was the intention of the present study to investigate this possibility by the method of delayed supplementation of tryptophane. One of the authors⁵ has shown that infantile rats, on a diet of incomplete amino acid mixtures, did not grow if the missing amino acid was fed separate from the rest of the diet several hours later. These experiments indicated that amino acids are not stored in the body, and that protein synthesis can proceed only when all essential amino acids are simultaneously present in the tissues.

If the delayed supplementation of tryptophane would prevent cataract formation with-

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¹ Chan, T. T., Chang, C. Y., and Luo, T. H., *Chinese J. Physiol.*, 1941, **16**, 241.

² Totter, R. J., and Day, P. L., *J. Nutrition*, 1942, **24**, 159.

³ Albanese, A. A., and Buschke, W., *Science*, 1942, **95**, 584.

⁴ Sydenstricker, V. P., Schmidt, H. L., Jr., and Hall, W. K., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 59.

⁵ Geiger, E., *J. Nutrition*, 1947, **34**, 97.

out promoting growth, we might conclude that this amino acid exerts its protective action independent of protein synthesis. Such a possibility has been suggested by Cahill and Kohalik⁶ who found that abrine (1-methyl-tryptophane), which is not as efficient as tryptophane in promoting growth, nevertheless prevents cataract in animals on tryptophane free diets.

The experiments were performed on infant rats (35-45 g body weight) in 2 groups of 6 each. The first group was fed the tryptophane deficient Diet I for 12 hours, and Diet II, containing tryptophane, for the next 12 hours. The second group received Diet I for 2 hours and Diet II for 2 hours; all food was withdrawn for the duration of one hour when diets were changed in this group. For further technical details see Geiger.⁵

Composition of Diet I—30 g acidhydrolysed fish protein—70 g basal diet.

Composition of Diet II—6 g 1(-) tryptophane—94 g basal diet.

The basal diet contained: corn starch, 3050 g (78.3%); rice bran concentrate, 400 g (10.26%); cottonseed oil, 200 g (5.13%); U.S.P. salt mixture, 200 g (5.13%); fish oil (one g contains 2,000 I.U. vitamin A and 400 I.U. vitamin D) 50 g (1.27%); riboflavin, 75 mg; Ca pantothenate, 150 mg; choline chloride, 2.5 g.

The results regarding growth were essentially the same as those reported in the previous study. In spite of an adequate supply of tryptophane derived from the two diets consumed in 24 hours, the rats failed to show normal growth; this again demonstrated that tryptophane fed apart from the other amino acids could not be utilized for protein synthesis. We did not observe the alopecia or hyperexcitability described by Totter and Day.² These symptoms probably resulted from other nutritional lack (deficiency or certain vitamins, perhaps) of the diet employed by these authors.

The ocular findings were as follows. Lenticular involvement and corneal vascularisation was noted in 10 of the 12 experimental

animals. The 2 animals with no sign of cataract had been on the experimental diet for only 6 weeks. In all cases the lenticular opacities showed the character of a posterior cortical cataract. The opacities were first noted subcapsularly, but gradually involved most of the posterior cortex, and in 2 cases progressed to the anterior cortex, the nucleus, and finally the entire lens.

The shape of the opacities showed considerable variation. In one case, it involved the central portion of the posterior cortex, forming a flower shaped figure, while the peripheral region remained clear. In other cases, the peripheral margin developed denser opacities, while the central area remained clear. The suture lines became visible; dehiscence of the lines, fluid clefts and vacuoles became more and more noticeable. In a few cases the anterior cortex became involved, principally in the equatorial region. In 2 cases, this development was followed by progressive intensification of the milky, diffusely opaque appearance of the whole lens.

The period of development also showed marked variations. In most cases, some definite sign of cataract formation could be observed by the sixth week of the experiment. In the 2 cases where cataract development proceeded furthest, some incipient signs had been noted by the fourth week of the experiment with a rapid subsequent course. By the twelfth week the lenses appeared whitish and opaque to the unaided eye in these animals, while in other animals only posterior cortical opacities were observed.

These results are similar to, if not identical with, the growth and eye manifestations noted in the studies of rats on tryptophane deficient diets. There are no clinical differences between animals completely deprived of tryptophane and those receiving delayed supplementation with respect to corneal vascularization and cataract formation.

In these experiments, ocular symptoms developed in spite of the fact that the rats ingested relatively large quantities of tryptophane (0.1 to 0.2 g per day). Prevention of cataract, like promotion of growth, can be achieved only if tryptophane is supplied

⁶ Cahill, W. M., and Kotalik, G. C., *J. Nutrition*, 1943, **26**, 471.

simultaneously with the other essential amino acids. This suggests a possible causal relationship between disturbed protein synthesis and cataract formation. Further investigations as to why disturbance of protein synthesis by the lack of essential amino acids other than tryptophane and histidine does not result in cataract formation is required.

Summary. Experiments with delayed sup-

plementation of tryptophane show that cataract formation in rats occurs even after consumption of relatively large quantities of tryptophane. It seems that like growth promotion, cataract prevention by this amino acid can be accomplished only when it is supplied simultaneous with the other essential amino acids.

16075

Simulated Auricular Flutter in the Electrocardiogram of the Dog.

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In the course of studies with epinephrine in the unanesthetized dog, undulations of the baseline at a rate of the order seen in auricular flutter developed in the electrocardiographic tracing. At first sight, the mechanism appeared to be auricular flutter. The coincident occurrence of tachypnea and retching during the recording of these electrical phenomena suggested the possibility that these undulatory waves might be extracardiac, caused by retching and tachypnea.

The possibility exists that similar records in man might, on occasion, be misinterpreted as auricular flutter. It thus appears advisable to present these records for publication.

In Fig. 1A are shown two sections of a continuous recording of lead 2 obtained in the dog after the intravenous injection of one mg epinephrine. In the first few cardiac cycles of the top strip regular undulations in the baseline at a rate of 356 per minute resemble the "F" waves of auricular flutter. This was associated with tachypnea of the same rate. The ventricular rate is 65 per minute. In the middle of the tracing during a period of transitory apnea, the undulations are absent, no P waves are seen, and the contour and rate of the ventricular complexes

are unchanged (the QRS and T waves are huge and upright). Toward the end of the top strip the phenomenon in the first part of the tracing recurs and continues during the first part of the second strip which was recorded 2 minutes later. In the middle of the second strip it is possible to identify P waves even though they are partially obscured by the undulations. Some of these are labeled. The ventricular rate at this time is 88 per minute and irregular. We are dealing here with a nodal rhythm followed by a sinus arrhythmia at a faster rate and the record is obscured by extracardiac artefacts simulating impure auricular flutter.

Fig. 1B is a tracing of the femoral arterial pressure in the same dog recorded with a Hamilton manometer simultaneously with a pneumogram obtained with a pneumograph strapped around the animal's chest. Below is a baseline recording time in seconds. The pneumogram shows post-epinephrine tachypnea at a rate of 306 per minute. In some instances, not seen here, these respiratory waves may even appear as noticeable fluctuations of the blood pressure within a single cardiac cycle.

Fig. 2 is a continuous electrocardiographic tracing (lead 2) obtained under similar circumstances in another dog. At the beginning

* The department is supported in part by the Michael Reese Research Foundation.

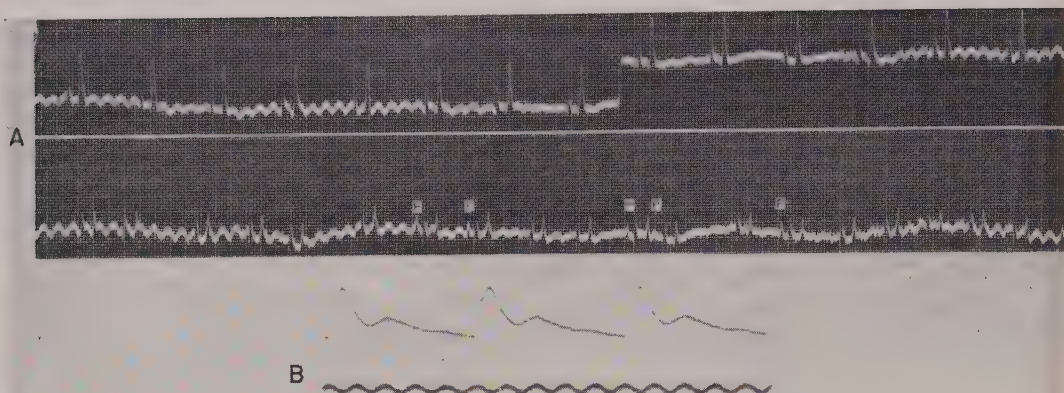


FIG. 1.

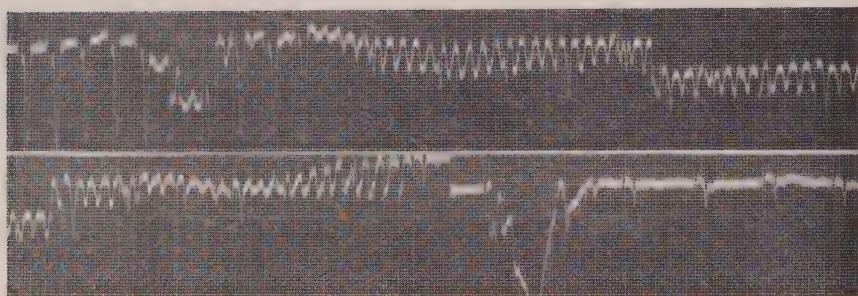


FIG. 2.

of the record ventricular tachycardia with a rate of 150 per minute is present with upright and prolonged QRS complexes (going off the record) and with deeply inverted T waves. Undulations of the baseline with a rate of 333 per minute begin in the middle third of the record and continue to the middle of the second strip. At times, they attain a huge size. When these undulations first appear, the ventricular rate slows abruptly to about 65 per minute, the configuration of the ventricular complexes changes, and the QRS becomes shorter in duration. No P waves are seen even at the end of the second strip when the undulations have disappeared. The baseline

undulations were observed to have occurred when the dog began to retch and vomit and are therefore probably attributable to artefacts caused by diaphragmatic movements (extracardiac artefacts). It appears that the ventricular tachycardia present at the beginning of the record broke at the onset of retching and was followed by A-V nodal rhythm.

Conclusions. Baseline movements which simulated impure auricular flutter developed following intravenous epinephrine injections. These are shown to be extracardiac in nature. They are induced by extreme tachypnea and the diaphragmatic movements associated with retching and vomiting.

Influence of Pamaquine and Atabrine on the Enzymatic Degradation of Quinine.

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The purpose of the present investigation is (1) to devise a procedure for the quantitative evaluation of the activity of the enzyme which destroys quinine and (2) to study the effect of pamaquine and atabrine on the degradation of quinine by the enzyme.

Materials and Methods. Suspensions of fresh rabbit's liver were prepared by homogenization with Ringer-Locke solution in a Waring blender. They were centrifuged at 1200 r.p.m. for 15 minutes; the supernatant only was used in the following experiments. The mixture and the supernatant were found to give the same enzyme activity and the same relationship between concentration and effect. However, more consistent results were obtained with the latter.

The enzymatic digestion was conducted at 39°C in an incubator. The enzyme preparation and the quinine solution of Ringer-Locke were kept in an incubator for half an hour before mixing. As a rule, 0.5 ml of the former was pipetted into 3.5 ml of the latter in a 30 ml centrifuge tube. At appropriate times equal volume of 4% NaOH was added to the digestion medium to terminate the reaction.

The determination of quinine was carried out by a method which has previously been described.¹ In brief, quinine was extracted from the digestion medium at an alkalinity of 2% NaOH with ethylene dichloride. In control experiments, with and without heating on a steam bath for an hour, the amount of quinine extracted with ethylene dichloride at this concentration of NaOH was found to be the same. The concentration of quinine was measured fluorometrically with the Coleman

photofluorometer (model 12). Pamaquine does not give any fluorometric reading with B₁, PC₁ filters. Atabrine shows a slight fluorescence through these filters; it does not, however, interfere with quinine determination. A blank value for atabrine is subtracted from the total fluorometric readings when quinine is determined in the presence of atabrine.

Results and Discussion. The graphs in Fig. 1 indicate the relationships between the percentage degradation of quinine and the concentration of liver. The rate of the reaction is shown by curves in Fig. 2. Except for low concentrations and during the beginning of the experiment, the degradation of quinine is in a linear relationship with the concentration of liver or with the time of reaction in logarithms. The linearity in rate was found to

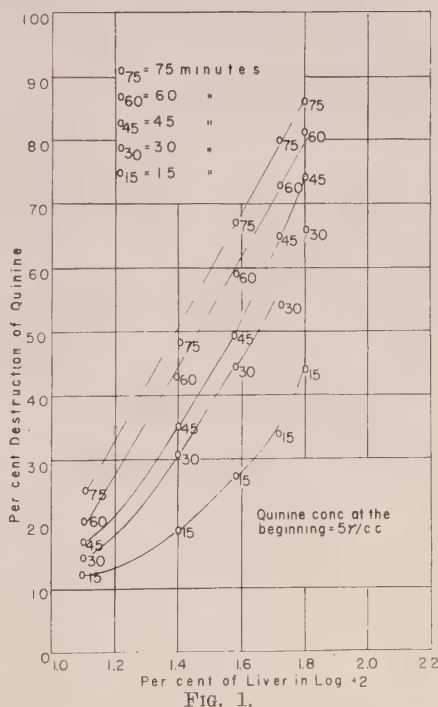


FIG. 1.

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¹ Chen, G., and Geiling, E. M. K., *J. Pharm. and Exp. Therap.*, 1944, **82**, 120.

hold as long as 8 hours for a digestion medium containing 5 $\mu\text{g}/\text{ml}$ of quinine and 0.25% of liver. Whether or not this relationship holds true for the pure enzyme and quinine awaits confirmation. If so, it may reveal the reaction mechanism of the enzymatic process.

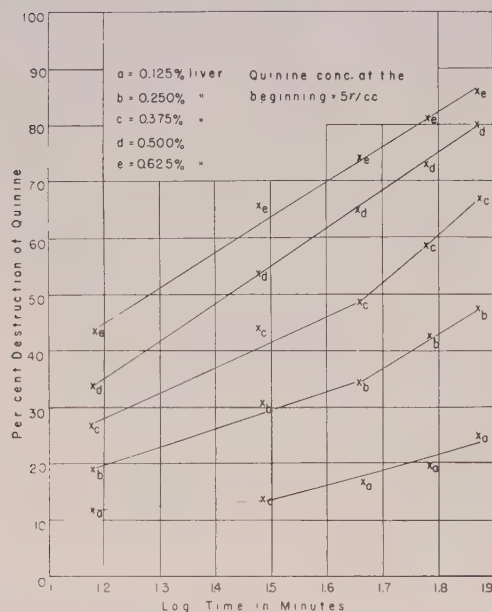


FIG. 2.

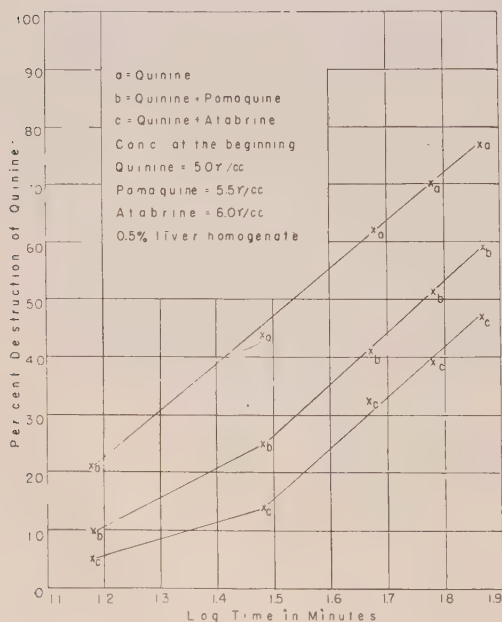


FIG. 3.

Clearly the linear relationship between concentration and effect for a certain duration of reaction, as shown in Fig. 1, may be utilized to determine quantitatively the enzyme activity of a preparation. The duration of one hour was chosen in the present work. The relationship deviates from linear at shorter intervals. The line for one hour will be taken as a reference standard in subsequent calculations for the enzyme activity of a sample by locating the corresponding value on the abscissa to the per cent degradation of quinine on the ordinate. For convenience in calculations, the value one on the abscissa will be considered as 10 units of enzyme activity.

The effect of pamaquine and the effect of atabrine on the enzymatic destruction of quinine were carried out under identical conditions as for the experiment in obtaining the reference standard; *i.e.* in a medium containing the supernatant of a 0.5% liver homogenate, 5 μg per ml of quinine and a molecular equivalent quantity of pamaquine or atabrine. Data in Fig. 3, obtained with the same sample of liver homogenate, indicate that the enzymatic destruction of quinine was decreased by the addition of pamaquine or atabrine. There is, however, no difference in the rate of the reaction. By interpolation from the line for one hour in Fig. 1, the enzyme activities as represented by the curves in Fig. 3 for the same period are as follows: for quinine alone, 50.1 units; quinine plus pamaquine, 30.2 units and for quinine plus atabrine, 22.4 units. The suppression of enzyme activity by pamaquine or atabrine is thus 39 and 54% respectively. It is not understood whether the effect of these two compounds is on the enzyme or on the substrate in the enzyme system. As judged by the rate of the reactions with and without pamaquine or atabrine, the effect of the two compounds is probably on the enzyme.

Summary. A procedure has been devised for the quantitative evaluation of the activity of the enzyme which degrades quinine. Pamaquine and atabrine were found to suppress the enzymatic degradation of quinine by a liver homogenate.

Urinary Excretion of Amino Acids by the Rat.*

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The adaptation of microbiological methods for the determination of amino acids in urine¹⁻⁸ has afforded considerable information on the levels of various amino acids excreted by several species as influenced by the amounts ingested. A paucity of information is available, however, on the influence of dietary deficiencies, such as specific amino acid or vitamin deficiencies, on the excretion of amino acids in the urine. In earlier work^{2,9} it was shown that vitamin B₆ deficient rats metabolized tryptophane to microbiologically inactive products at a rate comparable to that observed for vitamin B₆ supplemented animals. In the present study the effect of the ingestion of tryptophane deficient diets and the ingestion of diets deficient in riboflavin or nicotinic acid on the amounts of certain amino acids excreted in the urine was determined.

Experimental. Care of animals and composition of diets. Weanling rats were housed in single cages and were fed the experimental

diets for varying periods of time. Variations in the composition of the diets used were either in the level or kind of protein or in the B vitamin supplement added. Corn oil was added to all rations at a level of 4.7%, vitamin A and D concentrate 0.3%, salt mixture 4.0% and sucrose to 100%. Unless indicated otherwise, each diet contained 250 μ g each of thiamin and pyridoxine, 300 μ g of riboflavin, 2 mg of calcium pantothenate, 100 mg each of choline and inositol and one mg of nicotinic acid per 100 g. The diets were fed *ad libitum*. A brief designation of the diets is as follows:

No. 7—24% casein.

8—Same as No. 7 except no riboflavin was added.

9—12% casein.

10—12% casein and 12% gelatin.

11—Same as No. 10 except no nicotinic acid was added.

12—12% oxidized casein† plus adequate quantities of cystine and methionine. This ration was designed to be deficient in tryptophane.

13—Same as No. 12 plus 0.2% *dl.*tryptophane.

† The oxidized casein used in this investigation was prepared by the method of Toennies, *J. Biol. Chem.*, 1942, **145**, 667.

Urine collections were made by the use of metabolism cages fitted with outside feeders. The urine was collected during a 2- or 3-day period and food consumption records were also kept for these periods. Collections were made at least twice during the experimental period and data on at least two animals were obtained for each series of collections. Thus data on the amino acids excreted were obtained at different times throughout the experimental period. The urine samples from each group were combined prior to analysis so that data were obtained on 4 or more rats in each group. The collections were made at the same time for each series of deficient and sup-

* Frances Panzer and Helen Keene assisted with the amino acid analyses.

¹ Schweigert, B. S., Sauberlich, H. E., and Elvehjem, C. A., *Science*, 1945, **102**, 275.

² Schweigert, B. S., Sauberlich, H. E., Elvehjem, C. A., and Baumann, C. A., *J. Biol. Chem.*, 1946, **164**, 213.

³ Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1946, **166**, 417.

⁴ Steele, B., Sauberlich, H. E., Reynolds, M., and Baumann, C. A., *J. Nutrition*, 1947, **33**, 209.

⁵ Harvey, C. C., and Horwitt, M. K., *Fed. Proc.*, 1947, **6**, 259.

⁶ Hier, S. W., and Bergeim, O., *Fed. Proc.*, 1947, **6**, 261.

⁷ Frankl, W., and Dunn, M. S., *Arch. Biochem.*, 1947, **13**, 93.

⁸ Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *Arch. Biochem.*, 1947, **13**, 207.

⁹ Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

TABLE I.
Effect of Feeding Diets Low in Tryptophane on Urinary Excretion of Certain Amino Acids.*

Diet No. and description	Time on experiment, per day, days	Wt change per day, g	Histidine		Arginine		Threonine		Phenylalanine		Tryptophane	
			Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %
No. 12 (12% oxid. casein basal)	7-14	-0.9	0.15	2.1	0.28	2.3	0.17	1.4				
No. 13 (12% oxid. casein + tryptophane)	7-14	+2.2	0.13	0.95	0.16	0.73	0.14	0.64				
No. 12 (Group 2)†	4-10	-1.5	0.22	1.4	0.24	1.0	0.36	1.5	0.14	0.50	0.037	—
No. 13 (Group 2)†	4-10	+1.0	0.36	0.98	0.56	1.0	0.43	0.76	0.59	0.89	—	—
No. 7 (24% casein)	14-26	+4.4	0.25	0.34	0.57	0.64	0.54	0.48	0.36	0.27	0.15	0.41
No. 9 (12% casein)	14-26	+3.5	0.22	0.59	0.58	1.0	0.30	0.52	0.36	0.45	0.07	0.37

* Since the amounts excreted per day were very similar for urine collections made at different times during the experimental period, the values indicated are the averages for all collections made during the experimental period. The weight change was computed for only that period during which urine collections were made.

† Rats with an initial weight of 150 g were used in these experiments.

TABLE II.
Excretion of Amino Acids by Riboflavin and Nicotinic Acid Deficient Rats.*

Diet No. and description	Time on experiment, per day, days	Wt change per day, g	Histidine		Arginine		Threonine		Phenylalanine		Tryptophane	
			Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %	Amt excreted, daily, mg	Ingested amino acid excreted, %
No. 7 (riboflavin supp.)	14-54	+3.1	0.17	0.25	0.48	0.46	0.68	0.65	0.40	0.32	0.18	0.53
No. 8 (riboflavin def)	14-54	+0.3	0.22	0.67	0.64	1.3	0.59	1.17	0.45	0.76	0.14	0.85
No. 10 (12% casein, 12% gelatin + N.A.)	14-45	+2.8	0.37	0.77	1.41	0.80	0.81	0.92	0.46	0.47	0.13	0.66
No. 11 (12% casein, 12% gelatin + N.A.)	14-45	+0.4	0.18	0.75	0.61	0.68	0.32	0.72	0.28	0.55	0.058	0.58

* Since the amounts excreted per day were very similar for urine collections made at different times during the experimental period, the values indicated are the averages for all collections made during the experimental period. The weight change was computed for only that period during which urine collections were made.

plemented groups. The urine samples were stored in a refrigerator prior to analysis.

Amino acid analyses. Histidine, arginine, threonine, phenylalanine and tryptophane determinations were made after neutralization and proper dilution of the urine samples. These amino acids were chosen for analysis since it seemed likely that amino acids of this type may be more difficult to metabolize than the simpler amino acids. In addition, the methods for determining these amino acids in urine are quite accurate while the reliability for others is somewhat uncertain. *S. faecalis* R was used as the test organism for the determinations of threonine, arginine and histidine. The medium used has been described previously.¹⁰ Tryptophane and phenylalanine were determined with *L. arabinosus* as the test organism.^{1,11}

In some cases the occurrence of bound forms of the amino acids was investigated. Aliquots of the urine samples were hydrolyzed with one N HCl at 15 pounds pressure for 5 hours, neutralized, diluted to an appropriate volume and assayed for amino acids. By this procedure, data were obtained on the amounts of certain amino acids that were excreted in combined form, since the same samples were analyzed for amino acids prior to hydrolysis.

Results and Discussion. The amounts of histidine, arginine, threonine, phenylalanine and tryptophane excreted per day and the percentage of each amino acid ingested that was excreted in the urine are shown in Tables I and II. The latter figures were computed on the basis of the amount of each amino acid consumed and of the amount of each amino acid excreted per day. The composition accepted for casein was: histidine 2.6%, arginine 3.6%, threonine 4.0%, phenylalanine 4.7% and tryptophane 1.2%. For gelatin the values used were 0.6% histidine, 7.8% arginine, 1.8% threonine, 2.0% phenylalanine and 0.2% tryptophane. The amounts in casein and gelatin were determined by

microbiological methods. From these data and from the food consumption data the amounts of each amino acid ingested per day were calculated.

With the methods employed, the values for urine shown in Tables I and II represent the amount of each amino acid present in the urine that is microbiologically available. These values have also been termed the amount of apparent free amino acid present. Undoubtedly, the free amino acids comprise the largest portion measured, but it is recognized that certain amino acids present as peptides are utilized to some extent by the test microorganisms.¹² In order to determine the total amount present, it is necessary to hydrolyze the urine samples to liberate the bound forms of the amino acids.

It will be noted that even when rations deficient in an amino acid or a vitamin are fed for considerable periods of time, the amounts of the amino acids excreted per day are not materially different than those excreted by the control animals. Similarly, no difference was observed in the amounts excreted when determinations were made at different times in the experimental period. The percentage of the ingested amino acids excreted in the urine was somewhat higher, however, for the tryptophane or riboflavin deficient groups than for the respective control groups. The differences in the percentages of the ingested amino acids excreted were not as consistent, however, for the older rats fed the tryptophane deficient and supplemented diets (Table I). Rats fed a nicotinic acid deficient ration excreted approximately the same percentage of these amino acids as did the controls. In general, the amounts of each amino acid excreted when the control diets were fed (No. 7, 9, 10 and 13) were roughly proportional to the amino acid intake. The amount of arginine excreted, for example, was much higher when ration 10 (12% casein + 12% gelatin) was fed than when ration 9 (12% casein) or ration 7 (24% casein) was fed. This is undoubtedly due to the higher amounts of arginine ingested when ration 10

¹⁰ Greenhut, I. T., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **162**, 69.

¹¹ Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, 1944, **155**, 183.

¹² Schweigert, B. S., and Snell, E. E., *Nutr. Abs. and Rev.*, 1947, **16**, 497.

was fed since this ration contains gelatin which is very high in arginine.

Thus it appears that the deficiencies studied do not markedly affect the ability of the rat to metabolize the ingested amino acids to microbiologically inactive products even when the animals are losing weight at a rapid rate. It is quite possible, of course, that prolonged or chronic deficiencies may cause the rat to excrete much larger quantities of these amino acids. It is also possible that the metabolism of other amino acids not studied was materially affected. Some tests conducted with lysine and methionine indicate that they also were excreted in small amounts when diets deficient in tryptophane or riboflavin were fed. Preliminary studies indicate that vitamin B₆ deficient rats do not show large changes in the amounts of these amino acids excreted. These results are quite different from those observed with the mouse^{13,14} in that this species excretes large amounts of certain amino acids when deficient diets are fed. Thus an average of 37.3% of the ingested amino acids was excreted by mice when fed a tryptophane or methionine deficient diet while an average of only 2.6% was excreted when adequate diets were fed. Differences in the percentage of the ingested tryptophane that was excreted when normal diets were fed to rats as compared to mice have been discussed in earlier work.²

Data obtained on the amounts of histidine, arginine, threonine and phenylalanine determined after acid hydrolysis of the urine samples support the same conclusions as those on the amounts of microbiologically available amino acids (Table III); namely, that the amounts of these amino acids excreted are not appreciably affected by the ingestion of deficient diets by the rat. The results show that about $\frac{1}{4}$ to $\frac{1}{2}$ of the total amount excreted is measured as the free amino acid. These results are in agreement with those reported by Sauberlich and Baumann³ and Albanese *et al.*¹⁵

¹³ Sauberlich, H. E., Pearce, E. L., and Baumann, C. A., *Fed. Proc.*, 1947, **6**, 420.

¹⁴ Pearce, E. L., Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1947, **168**, 271.

TABLE III.
Effect of Acid Hydrolysis on Amount of Certain Amino Acids Found in Urine.* (All values expressed as mg excreted per day.)

Diet No.	Dietary regimen	Histidine		Arginine		Threonine		Phenylalanine	
		before	after	before	after	before	after	before	after
7	(24% casein)	0.25	1.4	0.64	1.6	0.54	1.5	0.36	1.6
9	(12% casein)	0.22	0.72	0.58	1.3	0.30	1.0	0.36	1.1
10	(12% casein, 12% gelatin)	0.37	1.2	1.41	3.0	0.81	1.7	0.46	1.8
11	(12% casein, 12% gelatin — N.A.)	0.18	0.78	0.61	1.8	0.32	0.83	0.28	1.3
12	(12% oxid. casein — tryptophane)	0.15	0.50	0.28	0.44	0.17	0.60	—	—
13	(12% oxid. casein + tryptophane)	0.13	0.36	0.16	0.42	0.14	0.35	—	—

* The values were obtained on the same samples before and after acid hydrolysis.

Although measurements of the amino acids in the urine and balance studies to ascertain the amount of any amino acid that is retained by the body are of great value, it should be recognized that alterations in the degree of metabolism of any amino acid are not necessarily determined by the microbiological methods used. Thus, when diets deficient in a specific amino acid or a vitamin are fed and no large differences in the excretion of the unchanged amino acid is observed, it may be quite possible that the amino acid is only partially metabolized and these partial metabolites excreted in the urine are microbiologically inactive. That marked changes in the amounts of the amino acids excreted in the urine do occur with mice fed various deficient diets has already been observed. Further studies on the specificity of these amino

acid methods and their reliability with respect to blood and urine analysis will aid in obtaining more information on the metabolism of specific amino acids by various species.

Summary. Rats have been fed diets deficient in tryptophane, riboflavin or nicotinic acid and the amounts of microbiologically available histidine, arginine, threonine, phenylalanine and tryptophane excreted in the urine were determined. No large changes were noted in the amounts of these amino acids excreted in the urine when rations deficient in nicotinic acid were fed. Rats fed a riboflavin or a tryptophane deficient diet excreted approximately twice as much of the ingested amino acids as animals fed adequately supplemented diets. In all cases, however, the amounts excreted were small, less than 2.5 per cent of those ingested. After acid hydrolysis of the urine samples, a 2-4 fold increase was observed in the values for histidine, arginine, threonine and phenylalanine.

15 Albanese, A. A., Holt, L. E., Frankston, J. E., and Irby, V., *Fed. Proc.*, 1946, **5**, 118.

16078

Vasoconstriction Elicited by Addition of Plasma to Vasoinactive Tissue Extracts.*

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Perfusion experiments on the rabbit's ear have shown that several vasoinactive, renin-containing protein fractions of hog kidney became vasoconstrictive when the perfusion fluid (Ringer-Locke) was supplemented by minute amounts of epinephrine.¹

Identical effects were observed when the kidney fractions were replaced by similarly prepared fractions of other organs—especially liver.²

A change from vasoinactivity to vasocon-

striction in the rabbit's ear was previously reported³ when plasma instead of Ringer-Locke was used for perfusion with kidney fractions (renin). This constriction was not attributed to the direct combined effect of the kidney fraction and plasma, but to the formation of a special vasoconstrictive substance, angiotonin (hypertensin).

Since plasma is known to contain traces of epinephrine,⁴ it seemed desirable to investigate whether this epinephrine content might have contributed to the constrictive effect elicited by kidney- or other tissue-fractions.

* Aided by a grant from the Commonwealth Fund.

¹ Mylon, E., Horton, F. H., and Levy, R. P., *Proc. Soc. Exp. Biol. and Med.* 1947, **66**, 375.

² Mylon, E., Horton, F. H., and Levy, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 378.

³ Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, **71**, 29.

⁴ Shaw, E. H., *Biochem. J.*, 1938, **32**, 19.

The following experiments were carried out to clarify this point.

Materials and Methods. Rabbit plasma was prepared as described by Landis⁵ with especial care in the heparinization of the animal 5 minutes before drawing the blood. This was then spun down at $+5^{\circ}\text{C}$ for 45 minutes, the plasma siphoned off and stored at the same low point until used. Fractions of liver extracts were prepared in accord with previously reported methods.² Epinephrine (Parke-Davis 1:1000) was freshly diluted several times a day to 1:100,000 with distilled water and used as stock solution. Further dilutions made from this concentration were used within 2 minutes. The subthreshold dose was usually between 1 cc and 1.5 cc of a 1:5,000,000 solution. The isolated rabbit's ear was chosen for perfusion. The volume of each separate substance to be tested was brought up to a constant amount by the addition of Ringer-Locke.

Results. The plasma from 5 of the 25 normal rabbits used in this study was in itself slightly and transiently vasoconstrictive on perfusion. The 25% constriction for 2 minutes observed with these 5 plasmas increased to 80% for 8 minutes on the addition of liver extract. This liver extract was completely vasoinactive if used for perfusion without plasma. Nineteen of the remaining 21 plasma samples had no effect on the isolated vessels until the liver extract was added to the perfusate. The degree of vasoconstriction recorded with these 19 samples varied from 60 to 99%, lasting for 3 to 10 minutes. The plasma samples of the two remaining rabbits elicited strong vasodilatation if added to the Ringer-Locke perfusate. Addition of the liver preparation abolished this vasodilatation.

The next experiments were designed to compare the effects of plasma and epinephrine on perfusion fluids containing liver preparations. Various combinations and sequences were tried. In none was it possible to differentiate between the effects of epinephrine and plasma on the liver containing perfusate. Typical experiments are recorded in Fig. 1 and Fig. 2.

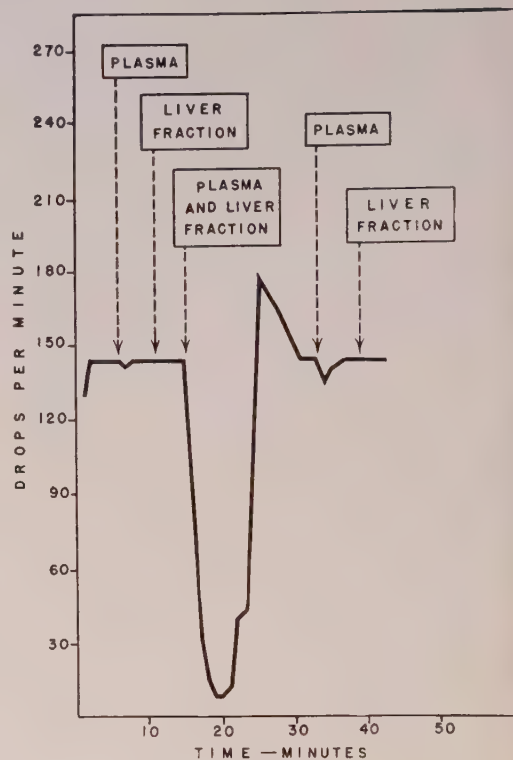


FIGURE-1
Vasoconstriction caused by the combination of vasoinactive plasma with a vasoinactive liver fraction.

All of the previous observations were carried out with fresh plasma that had been stored for not more than 3 hours at $+5^{\circ}\text{C}$.

In the light of the fact that the epinephrine content of plasma is destroyed on incubation,⁶ a new series of experiments was initiated in which the plasma was incubated for 12 hours before its use. The effect of this preliminary incubation on the plasma was striking: in none of the 8 experiments was any constriction obtained with plasma alone or in the combination of the incubated plasma with the liver fraction.

Discussion. The similarity of action of fresh plasma and subthreshold amounts of epinephrine suggests that the epinephrine content of the plasma is responsible for the observed effect on the rabbit's ear.

The colorimetrically determined values for "true" blood epinephrine, however, are smaller than the subthreshold doses that were used with the rabbit's ear.

⁵ Landis, E. M., Wood, J. E., Jr., and Guerrant, J. L., *Am. J. Physiol.*, 1943, **139**, 26.

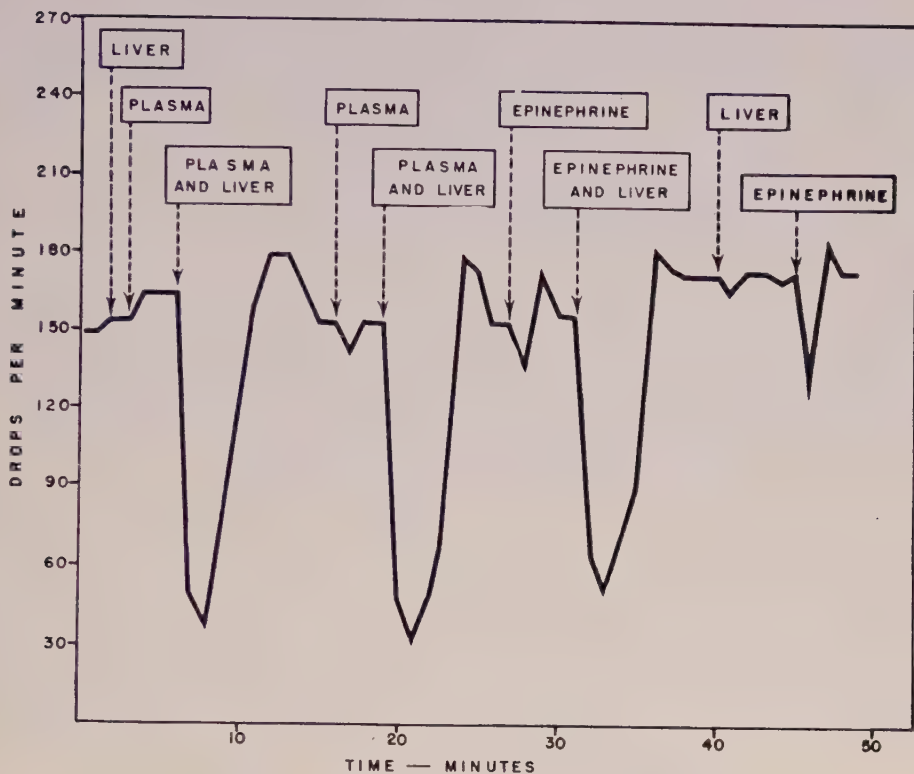


FIGURE II

Plasma or minute amounts of epinephrine if combined with a vasoinactive liver fraction act almost identically.

Recent studies^{4,7,8,9} indicating that the blood contains substances structurally related to epinephrine point to the possibility that these compounds are biologically active and involved in the reaction reported in this paper.

⁶ Bain, W. A., Gaunt, W. E., and Suffolk, S. F., *J. Physiol.*, 1937, **91**, 233.

⁷ Whitehorn, J. C., *J. Biol. Chem.*, 1935, **108**, 633.

⁸ Bloor, H. R., and Bullen, S. S., *J. Biol. Chem.*, 1941, **138**, 727.

⁹ Raab, H., *Biochem. J.*, 1943, **37**, 470.

Summary. Vasoconstriction of the isolated vessels of the rabbit's ear follows the addition of fresh plasma to perfusates containing liver preparations that are in themselves inactive. The activation caused by plasma is indistinguishable from the effect of subthreshold amounts of epinephrine added to these tissue fractions. Incubation of plasma, known to result in loss of its epinephrine content, also causes loss of its vasoconstrictive influence on perfusate containing liver fractions.

Deposition of Liver Glycogen in Normal Mice and in Mice Bearing Sarcoma 180.*

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(Introduced by C. P. Rhoads.)

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Previous investigation in this laboratory has demonstrated an impairment in the ability of patients bearing gastric cancer to lay down liver glycogen after the oral administration of glucose.¹ If small laboratory animals can be shown to be similarly affected by the presence of a tumor, more extensive and detailed studies of the abnormality would be possible. In animals the factors which normally influence glycogen storage can be better controlled. Moreover, the influence of tumor type and size as well as other variables pertinent to the study can be investigated more easily than is possible in human subjects.

This paper describes the results of measurements of liver glycogen in normal mice and in mice bearing sarcoma 180 implants before and after glucose administration.

Methods. Young, adult male mice, CFW strain, which were obtained from Carworth Farms, and which weighed from 16 to 26 g were used in all experiments. All animals were allowed drinking water *ad libitum* and were placed on a diet of Purina Laboratory Pellets for at least 2 days, to eliminate gross differences in nutritional state before use.

These animals were divided into 4 experimental groups. Two groups had had sarcoma 180 implanted into the axillary region 7 to 14 days previous to the experiment. Of these animals, one group provided fasting liver glycogen levels while the other group was treated with glucose before liver glycogen assay was made. The other 2 groups consisted

of normal mice. One group was fasted and one was given glucose. All animals were weighed frequently and on some occasions the food intake was followed. Except for those animals which had borne tumors for 12 to 14 days, all appeared healthy.

At 4 p. m. on the day preceding each experiment, the mice were deprived of food and placed in clean cages in groups of 2 to 4 in a dark, quiet, air-conditioned room.² After 16 hours, they were either sacrificed for the determination of fasting glycogen levels or were given an intraperitoneal injection of 100 mg of glucose (0.25 ml of 40% glucose in water) and sacrificed 2 hours later to allow measurement of the increase in liver glycogen over the control value. The mice were killed by severing the spinal cord at the base of the brain to minimize struggle. The whole liver was removed, weighed, and dropped into 30% KOH within 60 seconds of the time of death. Liver glycogen was determined by the Good, Kramer and Somogyi³ modification of Pfluger's method.

Results. As can be seen in Table I, the 16-hour fast sufficed to reduce the liver glycogen levels to a uniformly low level in both normal and sarcoma-bearing animals. The difference between the fasting levels in the groups of animals tested after bearing tumors for 7-10 days and 10-14 days is probably due to the small numbers of animals used and in any case is not significant in so far as this study is concerned, since these experiments were not designed to demonstrate differences in rates of glycogen depletion.

* This work has been supported by grants from the National Institute of Health of the United States Public Health Service, the Teagle Foundation, Inc., and the New York City Cancer Committee of the American Cancer Society, Inc.

¹ Abels, J. C., Young, N. F., and Homburger, F., in preparation.

² Swensson, Ake, *Acta Phys. Scand. Supp.*, 1945, 2, 33.

³ Good, C. A., Kramer, H., and Somogyi, M., *J. B. C.*, 1933, 100, 485.

TABLE I.
Deposition of liver glycogen

Group	Days after tumor implant	No. animals	Glucose, mg	Avg. body wt, gm	Liver glycogen, %	Total mg liver glycogen
Normal	—	27	0	19.6	0.29±0.02*	3.1
"	—	33	100	20.9	2.25±0.03	26.9
Sarcoma 180	7-10	5	0	18.0	0.19±0.02	2.3
" "	11-14	5	0	18.1	0.46±0.14	3.3
" "	7-10	17	100	20.4	1.15±0.08	14.4
" "	11-14	16	100	18.7	1.25±0.05	15.1

* Probable error of mean value.

All glycogen values are expressed in terms of glucose. Under the conditions of these experiments, the normal mice stored liver glycogen to the extent of 23.8% of the injected glucose as contrasted with only 12.0% by those animals with tumors. The probable errors of the means indicate that this is a highly significant difference whereas the difference between the mean values of the two tumor-bearing groups is not statistically significant.

Discussion. In view of the fact that, (a) the tumor-bearing mice did not lose weight; (b) their fasting liver glycogen concentrations were as high as those of normal mice; and (c) their food intake was as high as the normal, this difference can not be explained on a nutritional basis in the usual sense. Neither was the bodily activity of the tumor-bearing mice observed to be greater than that of the controls during the period between glucose injection and sacrifice. It is clear from the data that the length of time which

the mice have borne the tumors, at least between 7 and 14 days, has no influence on the glycogen content. Neither was the mass of the tumor found to influence the extent of glycogen storage. A group of animals bearing tumors having an average weight of 325 mg averaged 1.32% liver glycogen whereas a group with 1,200 mg tumors were found to average 1.24%. Greenstein⁴ found that the decrease in liver arginase and catalase activities of animals bearing tumors were likewise independent of the age of the tumor. It is possible that the impaired glycogenesis may result from a similar effect on the enzyme systems in the livers.

Conclusion. Male mice bearing sarcoma 180 implants exhibit an impairment in their ability to store liver glycogen after glucose administration. This is similar to the defect in patient with gastric cancer.

⁴ Greenstein, J. P., *J. Nat. Cancer Inst.*, 1942-1943, **3**, 419.

16080

Arginase and Catalase Activity in Livers of Patients Having Benign and Malignant Gastric Lesions.*

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The comparison of activities of certain

* This work has been supported by grants from the National Institute of Health of the United States Public Health Service, the Teagle Foundation, Inc., and the New York City Cancer Committee of the American Cancer Society, Inc.

enzymes of normal adult tissues with those of tissues undergoing rapid proliferation has revealed differences. These have given rise to much speculation regarding the possible relationship of enzyme activity to neoplastic growth. One important phase of this work

has been the demonstration that tumors may influence composition or metabolism of tissues not involved anatomically.

The most striking instance of such an effect is that reported by Greenstein.¹ He showed that the catalase activity of the livers of rats and mice bearing transplanted or spontaneous tumors was markedly decreased. On removal of the tumor, the activity promptly returned to normal, and a second transplant resulted in a second depression.² Similar although less marked effects were demonstrated on liver arginase activities in some species.³ These results lead one to conclude that the tumor may produce systemic effects in the host. Because of the implications of such a point of view, the extension of this type of investigation to the field of human cancer may be of great importance.

This paper presents the results of catalase and arginase determinations on liver biopsies obtained from 16 patients bearing gastric cancer and 11 patients who underwent surgery for benign lesions.

Clinical Material. The patients included in this study were unselected insofar as age, sex or nutritional state were concerned. They were all admitted to the Gastric Service of the Memorial Hospital for abdominal surgery and were given the usual pre-operative care and study. Food was withheld for at least 10 hours prior to operation. The liver biopsy was taken at the start of the operation, frozen within 15 minutes, and stored in a glass, airtight container at -40°C until immediately before analysis. The biopsy was invariably a wedge-shaped piece taken from the edge of the right lobe and weighed from 300-1000 mg.[†]

Methods. The weighed, frozen tissue was dropped into 6 volumes of ice-cold 0.5 N KCl solution, containing .03 N NaHCO_3

and thoroughly ground in a glass homogenizer. One milliliter of this mixture was diluted to 100 ml with cold distilled water and used immediately for estimation of catalase activity. The remainder of the homogenized tissue was allowed to incubate for 18 hours at 5°C before measurement of arginase activity.

Catalase activity was determined by estimating the H_2O_2 decomposed by 2 ml of the fresh, dilute tissue suspension added to 5 ml of approximately 0.09 M H_2O_2 (Merck's superoxol diluted 1-100) buffered with 1 ml M/5 sodium phosphate at pH 7.4. The mixtures were shaken for 20 seconds at 25°C and the reaction stopped by the addition of 2 ml 10 N H_2SO_4 . The remaining H_2O_2 was titrated with 0.04 N KMnO_4 and this value subtracted from a blank titration obtained when the acid was added before the tissue. Preliminary control experiments demonstrated the suitability of this test system with regard to linearity of H_2O_2 decomposed as a function of tissue concentration and of time. In no case was the availability of substrate a limiting factor.

The arginase estimations were performed exactly as described by Greenstein,⁴ except that the whole tissue *brei* was used rather than the supernatant extract. Ten dilutions of each activated extract were selected so that in the half hour incubation time chosen, the most dilute suspensions hydrolyzed from 8 to 20% of the arginase whereas the more concentrated suspensions effected complete hydrolysis.

The total nitrogen in each homogenate was determined by a micro-Kjeldahl method.

Results. Table I shows the results of the activity estimations together with pertinent data on each patient. Catalase activity is expressed as milliliters of oxygen liberated from the test system per milligram of tissue nitrogen per second. One unit of arginase activity is chosen as that which will effect the splitting of 50% of the arginine present in the test system in thirty minutes at 37°C . The arginase activities are expressed in units per milli-

¹ Greenstein, J. P., Jenrette, W. V., and White, J., *J. Nat. Cancer Inst.*, 1941, **2**, 282.

² Greenstein, J. P., and Andervont, H. B., *J. Nat. Cancer Inst.*, 1942, **2**, 345.

³ Greenstein, J. P., *J. Nat. Cancer Inst.*, 1943, **3**, 419.

[†] The cooperation of Drs. G. T. Pack and G. McNeer who, with their staff, made this study possible, is gratefully acknowledged.

⁴ Greenstein, J. P., Jenrette, W. V., Mider, G. P., and White, J., *J. Nat. Cancer Inst.*, 1940-1941, **1**, 687.

TABLE I.
Arginase and Catalase Activities of Human Liver Biopsies.

No.*	Sex	Age	Diagnosis	Catalase ml O ₂ /MgN/sec	Arginase AU/mgN†
82806	m	55	Gastric Ulcer	1.00	9.1
49722	m	64	" "	0.96	8.3
83610	f	48	" "	1.14	9.1
81622	m	45	" "	1.15	6.5
82072	m	53	" "	1.36	7.1
83417	m	43	" "	1.51	9.0
82719	m	48	" "	0.97	6.7
82233	m	59	" "	1.12	5.1
83114	m	50	" "	1.16	9.0
83584	m	53	" "	0.85	8.7
81603	f	48	" "	1.46	7.4
				avg 1.15	avg 7.8
82300	m	46	Prim. Op. Ca. Stomach	1.30	6.9
81764	m	64	" " " "	0.81	6.5
79547	m	45	Op. Ca. Duodenum	0.87	7.4
82108	f	60	Prim. Op. Ca. Stomach	0.74	7.1
82391	f	50	" " " "	0.93	6.1
81954	f	63	" " " "	1.12	8.0
82018	m	51	" " " "	1.28	7.2
83016	m	49	" " " "	0.94	6.7
83444	m	62	" " " "	1.58	10.0
83273	m	63	Inop. Ca. Stomach	1.06	15.0
81473	m	64	" " " "	1.26	4.8
80296	f	41	" " " "	1.04	10.2
75069	m	74	" " " "	0.86	7.1
25000	m	56	" " " "	0.42	4.4
80928	m	69	" " " "	1.02	6.5
80901	f	52	" " " "	0.78	5.0
				avg 1.00	avg 7.4

* Memorial Hospital Chart Number.

† AU = arginase activity which effects 50% hydrolysis under the conditions specified.

gram of tissue nitrogen.

It is apparent that there is no significant difference in arginase activity between the two groups of livers. The catalase values are quite variable in each group and although the average control value is 15% higher than that of the experimental group, the overlapping of the individual values is such as to make this difference an insignificant one.

Discussion. These negative results are of interest with respect to the work of Greenstein.^{1,2,4}

The negative results are also of interest with respect to the work of Abels⁵ and co-workers who demonstrated impairment of certain liver functions as an almost invariable concomitant of cancer of the gastro-intestinal tract in humans. Liver functions found to

be impaired included several which involve a variety of types of chemical reaction. Hence, it is of interest that the two functions chosen for this study should be found to be normal.

The decrease in arginase activity in the livers of animals bearing tumors may be limited to a few species since in no case were mice observed by Greenstein to exhibit this phenomenon. However, the decrease in catalase activities was found in both rats and mice with a wide variety of tumors but one exception. The actual magnitude of this decrease is not clear since later work by Greenstein indicates a 50% decrease in activity whereas in the first reports the decrease was 90% in many cases. In any event, it is clear that in humans the presence of adenocarcinoma of the stomach does not significantly influence the catalase content of the liver.

Conclusions. The catalase and arginase activities of liver biopsies from 16 patients

⁵ Abels, J. C., Rekers, P. E., Binkley, G. E., Pack, G. T., and Rhoads, C. P., *Ann. Int. Med.*, 1942, **16**, 221.

bearing gastric cancer did not differ significantly from those of 11 patients having benign gastric lesions. These results are in contrast to those found in animal experiments,

and are in contrast to those obtained with certain other liver function tests which appear to be impaired in patients with gastric cancer.

16081

Activation of Serum Protease in Peptone Shock.*

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A mechanism for the release of histamine and heparin in anaphylactoid conditions has been previously postulated by one of us.¹ According to this theory, the anaphylotoxins are released as a direct result of the activation of a serum protease which has been described in the literature as "plasma trypsin,"² "lytic factor,"³ "plasmin,"⁴ "tryptase"⁵ and "fibrinolysin."⁶ The activation of this protease in peptone shock is indicated by an increase in the fibrinolytic activity of the blood taken from dogs in the severe stages of shock, the fibrinolysis being observed in the tubes of the protamine titration test for heparin.⁷ Heparin has been shown to have an inhibitory effect on "plasma trypsin."² Hence the addition of protamine to the blood, as in the protamine titration test for heparin, provides conditions under which

the activation of the serum protease may be demonstrated by the resultant fibrinolysis.

Fibrinolytic activity of the blood has been observed by Nolf⁸ after the injection of peptone into the liverless and the anterior dog. Since the anterior dog has its inferior vena cava and thoracic aorta ligated just above the diaphragm, thus excluding the liver from the circulation, and since in the dog most of the heparin released comes from the liver, the anterior dog provides conditions *in vivo* similar to those provided *in vitro* by an excess of protamine. The anterior dog was used in our experiments to study the fibrinolytic activity of the blood in peptone shock. A second system which has been studied is the release of histamine from the leucocytes of the rabbit on the addition of peptone. The activity of the serum protease has been investigated by the use of the trypsin inhibitor isolated from unheated soya bean flour, which has been shown to be an inhibitor of the serum protease.⁹

Materials.

Soya Bean Trypsin Inhibitor (S.B.I.): We are indebted to Dr. M. Kunitz for a sample of this material recrystallized 5 times.

Peptone. Difco proteose-peptone.

Heparin (1000 units/ml) and **Protamine** (Salmine hydrochloride) were supplied by the Connaught Laboratories, University of Toronto.

* Aided by a grant from the John and Mary R. Markle, Jr., Foundation.

[†] Scholar of the National Council of Jewish Women of Canada for Research on Blood Plasma, University of Saskatchewan.

¹ Rocha e Silva, M., and Teixeira, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 376.

² Rocha e Silva, M., and Andrade, S. O., *Science*, 1945, **92**, 670.

³ Milstone, H., *J. Immunol.*, 1941, **42**, 109.

⁴ Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

⁵ Ferguson, J. H., *Science*, 1943, **97**, 319.

⁶ Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, in press.

⁷ Jakes, L. B., and Waters, E. T., *J. Physiol.*, 1941, **99**, 454.

⁸ Nolf, P., *Medicine*, 1938, **17**, 381.

⁹ Tagnon, H. J., and Soulier, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 440.

TABLE I.
Fibrinolysis of Blood of Anterior Dog in Peptone Shock.

Protamine (mg per ½ ml blood)	Minutes after peptone injection									
	0*		2		6		18		30	
	Hr	Units†	Hr	Units	Hr	Units	Hr	Units	Hr	Units
.100	4	2	1	4	2	3	2	3	2	3
.050	24‡	0	2	3	2	3	2	3	4‡	1
.025	"	0	2	3	2	3	2	3	4‡	1
.012	"	0	2	3	2	3	4‡	1	∞	0
.000	"	0	2	3	2	3	24	0.5	∞	0
Control	∞	0	2	3	2	3	24	0.5	∞	0
Total lytic activity		2		19		18		11		5

* After operation and before peptone injection.

† Arbitrary units, total lysis in 1 hr given the value 4, in 2 hr, 3, in 4 hr, 2, in 24 hr, 0.5, and partial lysis in 4 hr given the value 1.

‡ Only partial lysis (at least half the clot) in the time stated.

Fibrinogen. Armour Fraction I. *Thrombin.* Lederle's "Hemostatic Globulin."

Peptone Shock in the Anterior Dog. The dogs were anesthetized with sodium pentobarbital, arranged for recording carotid blood pressure and prepared as anterior animals according to Nolf.⁸ Injections of peptone were made into one of the external jugular veins and blood samples drawn from the other into a syringe rinsed with saline but containing no anticoagulant. The fibrinolytic activity of this blood was determined by adding it to a series of protamine tubes, the same as used in the protamine-titration test for heparin. A typical determination of fibrinolytic activity is shown in Table I. The fibrinolytic activity of each blood sample is expressed as the sum of the arbitrary units of activity assigned to each tube of the protamine series according to the length of time required for the lysis of the blood in that tube. The experiment recorded here shows the typical changes in fibrinolytic activity after peptone injection, an immediate increase followed by a slower decrease.

On the injection of peptone the blood pressure fell quite markedly in all cases but it was found that a concentration of 300 mg of peptone per kg was necessary to produce a prolonged fall in blood pressure and evidence of a prolonged activation of the serum protease.

To investigate the effect of the S.B.I. on the fibrinolysis in other experiments, part

of each blood sample was added to the S.B.I. solution and pipetted to the protamine series after the control. Since the S.B.I. has some anticoagulant activity⁹ a drop of thrombin was added to each tube of both control and S.B.I. series. The inhibitory effect of the S.B.I. on the fibrinolysis is well illustrated in Table II, where moderate lytic activity is completely inhibited by concentrations of S.B.I. as low as 0.34 mg per ml of blood.

In one experiment, S.B.I. (20 mg/kg) was injected into the anterior dog 2 minutes before the injection of the peptone. The injection of the S.B.I. had no effect on the blood pressure nor any effect on the fall in blood pressure after the injection of peptone. However, as shown in Fig. 1, there was a marked decrease in the degree of fibrinolysis. Also the preparation survived more than twice as long as a number of similar preparations subjected to the injection of peptone without previous treatment with S.B.I.

Since the platelets and leucocytes are believed to be involved in the production of peptone shock,¹ counts of the cellular elements were made during the experiments and are given in Table III. Platelets and leucocytes were greatly reduced after the injection of peptone, this reduction being accompanied by a marked agglutination of the platelets. The platelets and leucocytes decreased to the same level (approximately 25,000/mm³ for platelets and 2,000/mm³ for leucocytes) regardless of the dose of peptone, the initial

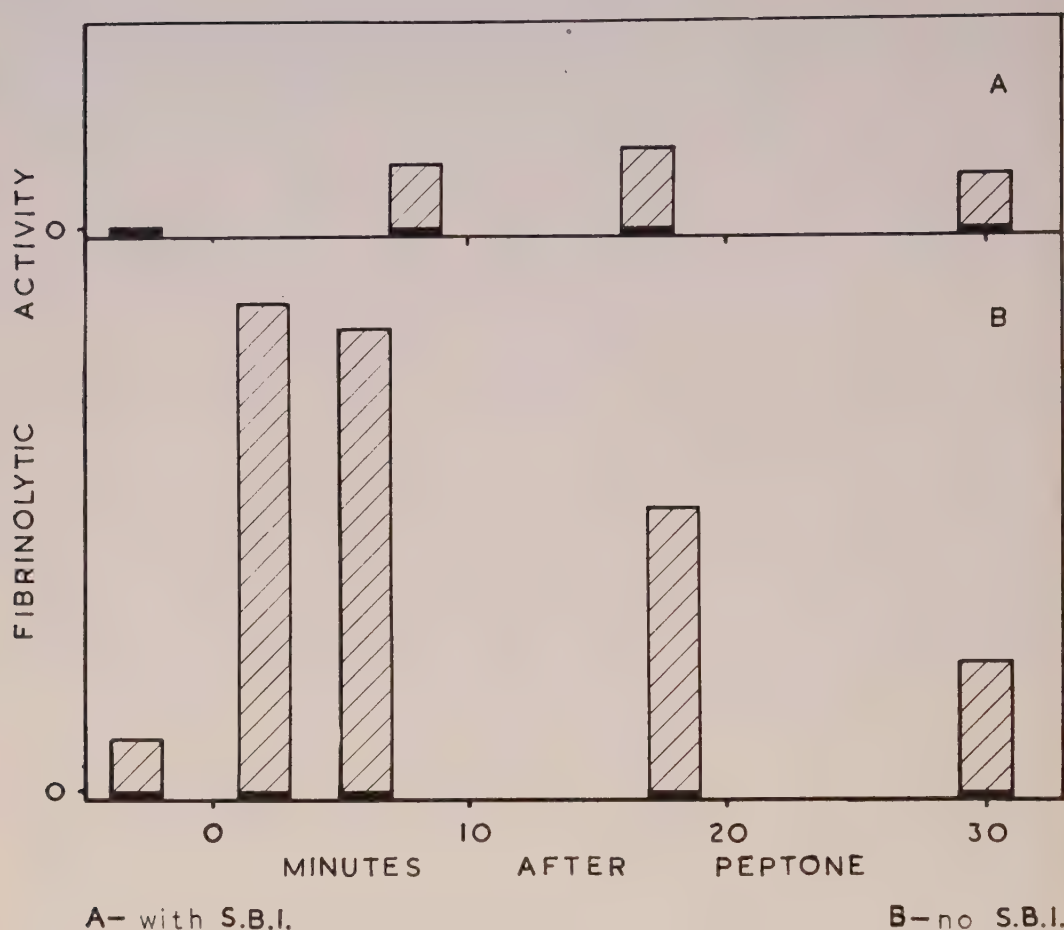


FIG. 1.

Inhibitory Effect of S.B.I. *in vivo* on Fibrinolysis. A—Anterior dog injected with S.B.I. (20 mg/kg) 2 minutes before the injection of peptone (300 mg/kg). B—Anterior dog injected with peptone (300 mg/kg).

level of the cells, the previous injection of the S.B.I., or the extent of the fibrinolysis produced.

The Effect of Protamine on Fibrinolysis. In peptone shock in the intact dog, the addition of protamine to the blood enables fibrinolysis to be observed. This could be due to the neutralizing effect of protamine on heparin, since heparin has been shown to antagonize the serum protease.² However, the fact that fibrinolysis occurred first in the tube with the highest concentration of protamine, regardless of the quantity of heparin present, indicated that protamine might have some other action promoting fibrinolysis as well as that of neutralizing

TABLE II.
Effect of S.B.I. on Fibrinolysis in Blood of Anterior Dog after Peptone.

S.B.I. (mg/cc blood)	Lytic activity of control blood	Lytic activity with S.B.I.
1.7	24	0
0.83	16	0
0.67	14	0
0.34	14	0
0.17	14	2

heparin. The proteolytic activity of the blood is thought to be inhibited normally by a proteolytic inhibitor which is present in serum, so that activation of the protease occurs when the inhibitor is removed. Protamine might act by competing with the free enzyme for

TABLE III.
Platelet and Leucocyte Count of the Anterior Dog in Peptone Shock.*

No. of dog	Dose of peptone (mg/kg)	Pre-operative		Before peptone		After peptone		
		Leucocytes /mm ³	Platelets × 1000/mm ³	Leucocytes /mm ³	Platelets × 1000/mm ³	Time min.	Leucocytes /mm ³ × 1000	Platelets × 1000/mm ³
3	150	12,100	419	10,300	440	6	2.2	28 (72%)†
						20	5.2	70 (50)
						35	4.6	69 (51)
4	200	17,600	258	11,300	276	6	2.4	26 (68)
						19	2.9	25 (30)
						58	7.5	85 (15)
						10	5.4	38 (33)
6	300	8,400	225	4,400	229 (9%)	5	2.0	19 (73)
						37	1.2	14 (45)
8‡	300	5,100	445	5,100	426 (6%)	8	2.0	22 (39)
						26	2.4	52 (42)
						11	2.5	35 (7)
10	300	15,900	481	11,200	471 (5%)	4	5.4	104 (76)
						30	3.7	19 (41)
						60	4.7	68 (33)

* Leucocyte and platelet counts have been adjusted to the red cell count in the normal sample.

† Figures in parentheses refer to the percentage agglutination of the platelets.

‡ Dog injected with 20 mg S.B.I. per kg 2 minutes before injection of peptone.

combination with the inhibitor released during the process of activation.

To study the effect of protamine on fibrinolysis, a lytically active dog serum was prepared by treatment with chloroform.¹⁰ This "chloroform serum" was used to lyse a standard fibrin clot, the lysis being inhibited by fresh dog serum which contains the natural inhibitor of the serum protease. The effect of protamine was tested on this lytic system, which, like the blood from the anterior dog in peptone shock, lacks the complicating factor, heparin.

In one experiment, a constant amount of protamine (300 µg) was incubated at 37.5°C with varying dilutions of the "inhibitor serum" for 5 minutes before the addition of the "chloroform serum" and clotting elements (0.2 ml of a 1:10 dilution of thrombin and 0.5 ml of 0.3% fibrinogen). Fig. 2 shows that for each dilution of the "inhibitor serum" the clot with protamine lysed before its control.

In another experiment, different amounts of protamine were incubated at 37.5°C with a constant "inhibitor serum" dilution for

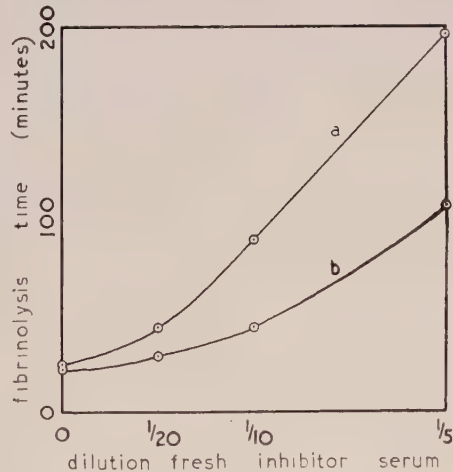


FIG. 2.

Effect of Protamine on Fibrinolysis. a (no protamine)—inhibitor serum + saline incubated at 37°C for 5 minutes, then 0.1 ml of chloroform serum, 0.2 ml of 1:10 thrombin and 0.5 ml of 0.3% fibrinogen added. b (+ protamine)—inhibitor serum + protamine incubated at 37°C for 5 minutes before the addition of chloroform serum + thrombin + fibrinogen.

5 minutes before the addition of the "chloroform serum" and clotting elements. Table IV A shows that increasing the concentration of the protamine in the clot increased the

¹⁰ Christensen, L. R., *J. Bacteriol.*, 1944, **47**, 471.

TABLE IV.
Effect of Protamine and S.B.I. on Fibrinolysis.*

A			B	
Protamine (μ g)	Inhibitor serum (1/5 dilution) (ml)	Lysis time (min)	S.B.I. (mg)	Lysis time
—	—	27	—	6.5 min
30	—	27	3.0	(no lysis in 12 hr)
—	.1	216	2.0	" " " " "
300	.1	138	1.0	" " " " "
30	.1	206	0.1	" " " " "
3	.1	2166	0.01	(lysis within 12 hr)
			0.001	" " " " "

* Standard fibrin clot containing 0.2 ml of a 1:10 dilution of hemostatic globulin and 0.5 ml of 0.3% fibrinogen (Armour Fraction I).

A—Total volume 1.5 ml containing 0.4 ml chloroform serum.

B—Total volume 1.4 ml containing 0.1 ml chloroform serum.

speed of lysis. S.B.I. added to a lytically active clot, inhibited the lysis, as shown in Table IVB. Tagnon⁹ has reported similar observations.

Effect of S.B.I. on release of histamine from rabbit blood by peptone. Blood was taken into heparin from rabbits by cardiac puncture. Part of the blood was incubated for 5 minutes at room temperature with S.B.I. (0.5 mg per ml of blood) while 2 controls were incubated with saline. To the blood + S.B.I. and to one of the controls, peptone was added (15 mg per ml of blood) while to the other control saline was added. The peptone used had been freed of histamine by treatment with permittit.¹¹ The mixtures were incubated for various times and histamine extractions performed on their plasmas by the method of Code.¹² The assay on the guinea pig ileum showed that in a number of experiments with an incubation time of 15 minutes, there was an inhibition by S.B.I. of the release of histamine by peptone. Little inhibition was demonstrated by the S.B.I. with an incubation time of 30 minutes.

The effect of time of incubation on the release of histamine is shown in Fig. 3. Three mixtures, a and c containing blood + saline and b containing blood + S.B.I., were incubated for 5 minutes at room temperature. Peptone was added to a and b and saline to c, the concentrations of S.B.I. and peptone

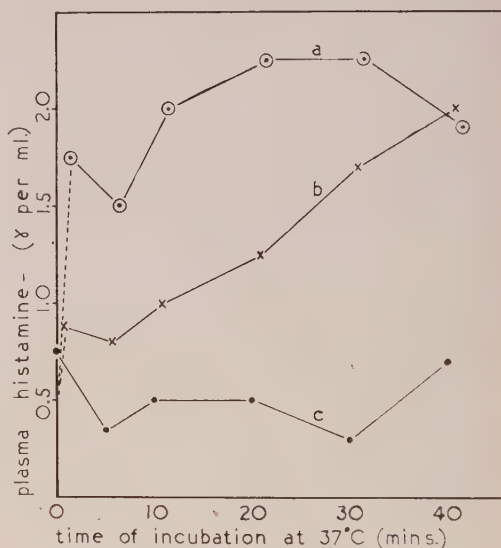


FIG. 3.

Effect of S.B.I. on the Release of Histamine from Rabbits' Blood by Peptone. a—Blood + Peptone. b—Blood + S.B.I. + Peptone. c—Blood + Saline.

in the blood being the same as above. From these mixtures incubated at 37°C, samples were taken at various times and packed in snow until all the samples were taken. These were centrifuged simultaneously and their plasmas extracted for histamine. With saline alone added to the blood, plasma histamine remained constant, the variation shown in the samples indicating the degree of accuracy of the method used ($\pm 0.2 \mu$ g histamine). With peptone alone added to the blood, the release of histamine initially was rapid, slowing to

¹¹ Gotz, F. R., and Dragstedt, C. A., *J. Pharm. and Exp. Ther.*, 1941, **74**, 33.

¹² Code, C. F., *J. Physiol.*, 1937, **89**, 257.

a plateau in about 20 minutes. When the blood had been incubated with S.B.I. previously, the addition of peptone was not followed by a rapid increase in plasma histamine. However, the S.B.I. did not completely inhibit the release of histamine, which proceeded at a slow rate.

Discussion. As shown above, we have found that S.B.I. inhibits fibrinolysis due to the serum proteolytic enzyme activated by chloroform, while protamine inhibits the natural serum inhibitor of this enzyme. Since S.B.I. inhibited the lysis occurring in peptone shock in the anterior dog, while the addition of protamine increased the lysis, it is evident that the fibrinolysis due to peptone is due to the activation of the plasma enzyme. S.B.I. inhibited the release of histamine by peptone from rabbit blood cells. This suggests that activation of serum protease is a step in the release of histamine by rabbit blood cells. Unfortunately sufficient S.B.I. was not available for the corresponding experiment in the dog to test the effect on liberation of histamine by body cells.

The action of the strongly basic protamine on fibrinolysis of both blood and fibrin clots lends support to the hypothesis of Ferguson⁵ and others that the inhibitor of the plasma proteolytic enzyme is a polypeptide with acidic groups analogous to those of heparin or that heparin is a prosthetic group for this inhibitor.

The strikingly uniform reactions of platelets and leucocytes, as far as their counts are concerned, despite wide variation in the fibrinolytic effect produced, would indicate that these cell elements are neither concerned with the production of, nor affected by, the

activation of the serum protease. However, there is some evidence that this may not be the case. Smears from the cut edge of fragments of lung tissue, prepared according to the technique described by Rocha e Silva¹³ showed that when fibrinolysis was marked, disintegration of the platelet clumps in the lung vessels had proceeded to a degree where only dark-staining granules remained as remnants of the disintegrated platelets. When fibrinolysis was weak or absent, the disintegration of the platelets in the clumps was very slight or absent. Thus it appears that although the counts of circulating platelets and leucocytes may not indicate any relationship to fibrinolysis, the smear technique for examining clumps of platelets and leucocytes may indicate the true relationship of these cell elements to fibrinolysis and other results of the injection of peptone.

Summary. The relationship of the serum protease to the action of peptone was investigated using soybean trypsin inhibitor and protamine. Soybean trypsin inhibitor inhibited fibrinolysis by chloroform serum (activated serum protease). It inhibited the fibrinolysis occurring in peptone shock in the anterior dog and also the release of histamine from rabbit blood cells *in vitro* on the addition of peptone. Protamine accelerated fibrinolysis in the anterior animal and also lysis by the serum protease in the presence of the natural serum protease inhibitor.

We wish to acknowledge the hospitality of Dr. C. H. Best in the Department of Physiology, University of Toronto. We are indebted to Dr. E. Fidler for the platelet counts.

¹³ Rocha e Silva, M., Porto, A., and Andrade, S. O., *Arch. Surg.*, 1946, **53**, 199.

Susceptibility of the Hamster to St. Louis and Japanese Encephalitis Viruses by Feeding.*

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It was reported previously¹ that hamsters, when allowed to feed upon St. Louis encephalitis virus, either die with encephalitis, or develop an immunity as evidenced by appearance of serum antibody and resistance to intracerebral inoculation. Subsequent tests have indicated that hamsters, fed Japanese encephalitis virus react in much the same way. The present report describes the extent of the infection in hamsters following feeding with St. Louis (Hubbard strain) and with Japanese (Nakayama strain) encephalitis viruses, as determined by subinoculation of mice with various tissues.

The possibility of infection by way of the alimentary tract with neurotropic viruses, other than those of poliomyelitis and encephalomyelitis of mice, has received some attention. Infection of mice by introduction of St. Louis virus into the alimentary tract has been described by 2 groups of investigators,^{2,3} and both report development of immunity in survivors. The latter group also fed 2 hamsters with St. Louis virus and found neutralizing antibody some days later.⁴

In our experiments hamsters were induced to eat virus by starving them overnight, and offering them the heads of infected mice. Tissues to be tested for virus were carefully

removed from exsanguinated animals with separate sets of instruments, emulsified in a minimum quantity of broth to which 10% normal sheep serum had been added, centrifuged, and the supernatant inoculated into 3 or 4 mice each. In the case of bacterially contaminated tissues such as the intestine, the specimens were washed repeatedly in sterile physiological saline and were thereby rendered sufficiently free of bacteria to allow intracerebral inoculation.

In most of the experiments a second intracerebral passage to mice was made with the brain tissue of any mouse found ill or dead, in which there was doubt concerning the cause. A simple technic, as described elsewhere,⁵ was used. Blood specimens were taken by cardiac puncture and immediately injected intracerebrally into mice.

Preliminary tests for St. Louis virus in hamster tissues included the large and small gut, the lymphoid follicles present in the wall of the latter, and the mesenteric lymph node. Virus was found on occasion in one or more of these tissues from the first to the ninth day after the hamster had fed upon virus but the incidence was greatest in the 2 lymphoid tissues on the fourth or fifth day. Three different strains of virus recovered in these experiments from intestine or associated lymphoid tissue of hamsters were identified as St. Louis virus in neutralization tests with immune rabbit serum.

Ten attempts to recover virus from intestinal contents of hamsters fed upon St. Louis virus included both filtered and unfiltered samples, but were unsuccessful.

The results of more extensive experiments are presented in Table I. It is evident that both viruses become widespread in the ham-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Gordon, F. B., *J. Bact.*, 1944, **47**, 465 (abstract).

² Harford, C. G., Sulkin, S. E., and Bronfenbrenner, J., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 332; Harford, C. G., and Bronfenbrenner, J., *J. Inf. Dis.*, 1942, **70**, 62.

³ Mezera, R. A., Broun, G. O., Muether, R. O., and LeGier, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **48**, 357.

⁴ Broun, G. O., LeGier, M., Mezera, R. A., and Muether, R. O., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **48**, 310.

⁵ Schabel, F. M., Jr., and Gordon, F. B., *Science*, 1947, **106**, 549.

TABLE I.
Distribution of St. Louis and Japanese Encephalitis Viruses in the Hamster After Feeding.

Tissue tested	St. Louis virus	Japanese virus
(3rd to 8th day)		
Buccal mucosa	3/10	2/10
Tongue	7/10	6/10
Larynx	5/10	5/10
Cervical lymph nodes	8/10	7/10
Esophagus	3/10	3/10
Stomach	0/10	0/10
Small gut	7/10	1/10
Lymphoid follicles of small gut	6/10	4/10
Large gut	6/10	3/10
Mesenteric lymph node	8/10	4/10
Inguinal lymph node	5/10	5/10
Nasal mucosa	4/10	8/10
Olfactory bulbs	3/10	8/10
Cerebrum	5/10	7/10
Brain stem	4/10	6/10
Spinal cord	4/10	6/10
Spleen	6/10	
Striated muscle	0/10	
(1st to 5th day)		
Blood	10/46	30/38

Denominator indicates number of tests made; numerator indicates number positive.

ster after feeding, being recoverable from many tissues, including the blood, a fact which obscures both the site or sites of multiplication of virus, and the sequence of events in the pathogenesis of the infection.

Viremia has been present as early as the second day after feeding with St. Louis virus, and the first day in the case of Japanese, but it is intermittent in the former case and with neither virus has it been demonstrable after the fifth day. The presence of virus in the blood and certain other tissues before it is recoverable in the central nervous system (3rd to 8th day) suggests that multiplication may occur outside the central nervous system.

Both of these viruses have been recovered by swabbing the mouths of hamsters on the sixth to ninth days after feeding, when clinical signs were present. The cotton swabs

were washed out in serum-broth, penicillin was added (2000 units per cc) and the supernate after centrifugation was injected intracerebrally into mice. By this technic St. Louis virus was recovered in 3 of 5 attempts in 3 hamsters, and Japanese virus in 12 of 13 tests on 12 hamsters. The latter virus has also been found in the mouth 7 times in 6 hamsters on the first to fifth day after feeding, before any clinical signs of infection were apparent. St. Louis virus was isolated from the mouths of hamsters (2 of 3 on third day; one of 2 on fourth day) that had been injected intracerebrally.

We are unaware of any direct evidence at present that transfer of virus to or from the alimentary canal is significant in the epidemiology of these encephalitides. It is of interest to note, however, that evidence has been submitted⁶ for the presence of St. Louis virus in the nasopharyngeal washings of patients and has been recovered from nasal washings of an intracerebrally inoculated horse.⁷ Venezuelan equine encephalomyelitis virus has been recovered from human nose and throat washings.^{8,9,†} It appears that the possibility of natural hosts of these viruses acquiring infection through the alimentary tract, and transmitting either by droplets from the mouth or by the feces, deserves careful consideration.

⁶ Sulkin, S. E., Harford, C. G., and Bronfenbrenner, J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 327.

⁷ Cox, H. R., Phillip, C. B., and Kilpatrick, J. W., *Pub. Health Rep., U. S. P. H. S.*, 1941, **56**, 1391.

⁸ Casals, J., Curnen, E. C., and Thomas, L., *J. Exp. Med.*, 1943, **77**, 521.

⁹ Lennette, E. H., and Koprowski, H., *J. A. M. A.*, 1943, **123**, 1088.

† There are also several pertinent references in the Japanese literature, the originals of which we have been unable to obtain.

Influence of Alpha Tocopherol on Implantation in Old Rats.*

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Investigations concerned with the fertility of female rats on vitamin E deficient diets have been mainly focused on the "resorption gestation." The question of implantation in vitamin E deficiency has received much less attention.¹

In these experiments, the rate of implantation of female rats at different age periods on a vitamin E deficient diet was compared with that of rats on the same diet completed by addition of alpha tocopherol.

Methods. 1130 mating experiments were carried out on 293 albino rats of a highly inbred colony. The ancestry of these rats had been kept for 7 or 8 generations on a modified "Evans-Burr" diet containing 20% commercial lard. When these females gave birth to a litter, they were immediately placed on a simplified diet on which they, their offspring and subsequent generations were kept without interruption.

The diet consisted of:

	parts
commercial lard	10
crude casein	30
cerelease	54
cellulose	2
salt mixture	4
	mg/kilo
pyridoxine	4
thiamine chloride	2
riboflavin	4
choline	1000
vitamin K	4
p-aminobenzoic acid	300
calcium pantothenate†	10
oleum percomorphum	200

* Aided by a grant from the John and Mary R. Markle Foundation.

¹ Emerson and Evans, *J. Nutrition*, 1939, **18**, 501.

† We are indebted to Messrs. James G. Blase and Nicholas F. Simeone of the Abbott Laboratories for the choline and part of the synthetic vitamins, and to Misses Kathryn Faraci and Marian Powelson for technical assistance.

Its tocopherol content was repeatedly checked according to the method of Kaunitz-Beaver² and was found to be below .4 mg per 100 g of diet. From these determinations and from those of Quaife and Harris,³ it can be estimated that the daily intake of the adult rats was in the neighborhood of 20-40 micrograms of tocopherols, because lard was the only measurable source of tocopherol in the diet. The latter was freshly prepared at least once weekly and kept under refrigeration. Five generations of rats raised on this diet were used for the tests. The diet had previously proved to be satisfactory in studies of growth and bioassays (Kaunitz;⁴ Kaunitz and Beaver⁵).

Controls derived from the same colony were kept on the same diet augmented by 4 mg synthetic dl alpha tocopherol acetate† per 100 g diet. The weights of these animals, charted according to Zucker and Zucker,⁶ were satisfactory. Females of the first through fifth generation were used approximately in the same proportion as those on the deficient diet.

Mating was carried out by leaving the female for 5 days with the selected male. On and subsequent to the 15th day after mating had begun, the females were examined for the "implantation sign" and their weight was taken on the same scale daily except for occasional omissions. If the implantation sign

² Kaunitz and Beaver, *J. Biol. Chem.*, 1944, **156**, 653.

³ Quaife and Harris, *Ind. and Eng. Chem. (Anal. Edit.)*, 1946, **18**, 707.

⁴ Kaunitz, *J. Nutrition*, 1946, **32**, 327.

⁵ Kaunitz and Beaver, *J. Biol. Chem.*, 1946, **166**, 205.

† Dr. Leo Pirk of Hoffmann-La Roche very kindly supplied us with alpha-tocopherol and part of the synthetic vitamins.

⁶ Zucker and Zucker, *J. Gen. Phys.*, 1942, **25**, 445.

TABLE I.
Mating Experiments on Rats on a Purified Diet Containing About 100-400 Micrograms Tocopherols per 100 g, According to Age and Tocopherol Supplement.

Alpha tocopherol acetate supplement	No. of experiments	No. of pregnancies	% Implantations	No. of Litters	No. of Resorptions	Litters in % of pregnancies
Age 53-150 Days.						
None	47	31	66	3	28	10
Single dose of .5-1 mg	48	36	75	9	27	25
" " " 1.1-2 "	51	40	78	23	17	58
" " " 2.1-3 "	11	9	82	8	1	89
4 mg per 100 g diet	55	48	87	48	0	100
Age 151-240 Days.						
None	53	29	56	4	25	14
Single dose of .5-1 mg	37	27	73	9	18	33
" " " 1.1-2 "	47	30	64	20	10	67
" " " 2.1-4 "	18	15	83	14	1	93
4 mg per 100 g diet	22	16	73	15	1	93
Age 241-360 Days						
None	22	4	18	2	2	9
Single dose of .5- 2 mg	24	16	66	4	12	25
" " " 2.1- 4 "	14	7	50	4	3	57
" " " 4.1-10 "	26	15	58	8	7	53
4 mg per 100 g diet	32	24	75	24	0	100
Age 361 Days and Over.						
Single dose of 0 - 3 mg	20	2	10	0	2	0
" " " 3.1-10 "	29	12	41	2	10	18
"Massive" doses	50	11	22	9	2	82
4 mg per 100 g diet	33	20	61	17	3	85

was followed by weight gain and thereafter by gradual weight loss, a resorption gestation was recorded. In 22 not entirely clear cut cases, autopsies were performed. A litter was registered regardless of the number of dead and living young. Animals which persistently failed to become pregnant were excluded from the charts in accordance with the practice of Evans and Burr.⁷ Only females of 150 g or over were used for breeding.

With this technique, pregnancy was observed in 36 out of 43 mating tests (84%) in rats on "Evans-Burr" diet, and in 87% of the younger rats on the complete diet. These data are in good agreement with Evans' and Burr's⁷ observation on the implantation rate in a rat colony kept on a complete diet, and with those reported by Goettsch and Pappenheimer.⁸

In the earlier experiments, males of proven fertility kept on Rockland rat diet were used. In over 900 of the experiments, however, the males raised on the complete simplified diet were mated. In case no pregnancy was ob-

served, the result was charted only if the male was fertile before and after the negative test.

The females were used repeatedly. On the 5th day after the mating had commenced and after the male had been removed, they were either left on the diet or they were given single supplements of alpha tocopherol acetate diluted with 30 g of the experimental diet. The cages were cleaned before the supplements were offered, and no additional food was given until the supplement was completely consumed, usually within 3 days. The single doses amounted to .5-3 mg alpha tocopherol during the first 5 months of life, to .5-4 mg from the fifth to the eighth months, and thereafter to .5-10 mg.

Fifteen female rats kept on the deficient ration, were given 1-2 drops (30-60 mg) of alpha tocopherol acetate at intervals of 1-2 weeks, after they had reached the age of one year.

After a resorption gestation or after the weaning of a litter (towards the end of the fourth week), at least 4 weeks elapsed before the female was mated again.

It is probable that some pseudopregnancies were recorded as resorptions, because the

⁷ Evans and Burr, *The Antisterility Vitamine Fat Soluble E*, Univ. Cal. Press, Berkeley, 1927.

⁸ Goettsch and Pappenheimer, *J. Nutrition*, 1941, **22**, 463.

nature of these experiments excluded verification by autopsy in the majority of the cases. A further source of error was the repeated administration of tocopherol to the same rat. Both these sources of error should reduce the number of rats in which failure of implantation was recorded. The statistical differences between the groups thus became even more significant.

Experiments. In Table I are computed the results of the mating tests in relation to age and tocopherol supplement. After the 240th day there is an abrupt reduction in the percentage of implanting in the animals without tocopherol supplements which is in agreement with Emerson's and Evans' observations. Single supplements of .5-2 mg after the eighth, but before the twelfth months, and doses of 3-10 mg after one year increased the rate of implantation significantly. The "mean fertility dose" of the animals of 8-12 months was roughly 3-4 mg, and after one year litters were observed only twice with single doses up to 10 mg.

When "massive doses" of tocopherol (30-60 mg in intervals of 1-2 weeks in addition to the "complete" diet) were administered to rats after at least one year on the deficient diet, about one-half of the animals gave birth to litters, but only after repeated matings. The implantation rate of the females that had received the diet plus alpha tocopherol throughout life was still practically normal at that time.

One group of 27 rats had been kept on Rockland rat diet during the first 2 months of life and thereafter on the experimental diet. At the age of 12 months, no implantations could be observed after repeated matings. When now a single dose of 6 mg alpha tocopherol acetate was offered after a single mating, litters were observed in about 2/3 of the experiments.

Discussion. Evans and Burr⁷ have discussed possible pitfalls in the evaluation of implantation data obtained from rats on vitamin E deficient diets. Failures may be caused by toxic substances in the diet or by deficiencies other than vitamin E. These sources of error can hardly explain the de-

crease in the implantation rate in these experiments, because appropriate single post mating supplements of tocopherol increased the percentage of pregnancies significantly, and breeding was normal when the diet was completed by alpha tocopherol.

Infections of the uterus and the tubes were frequently observed by us in the older rats on the deficient diet. Similar observations have previously been reported by Emerson and Evans.¹ In some instances, the uterus contained 5-10 cc thick purulent material. Purulent fistulas in the inguinal region, originating from uterine infections, were observed 4 times. No purulent infections were noted in the rats on the complete diet. While infection of the genital organs may have prevented pregnancies in some of the rats, its occurrence can be excluded in those rats which responded to single post-mating doses of alpha tocopherol. Infection of the uterus and the tubes would have interfered with both the fertilization and the implantation of the ovum.

The failure of implantation in the rats of over 8 months of age indicates that, in this age group, tocopherol is essential for the implantation of the previously fertilized ovum. The requirements appear to increase with age and there are great individual variations.

Animals that had been administered constant tocopherol supplements, had a practically normal implantation rate after one year of age; among the group receiving "massive doses" after one year or more on the deficient diet, about one-half of the rats gave birth to litters, but only when mating was carried out repeatedly; the same age group on the basic diet with single tocopherol supplements of 3-10 mg had frequent resorption gestations but only 2 litters. This indicates that the changes leading to failure of implantation are prevented but, once present, only partly cured by alpha tocopherol.

Summary. 1. The implantation rate in female rats on a purified diet containing about .1-.4 mg of tocopherols per 100 g diet was compared with the implantation rates in animals on the same diet receiving single and continuous supplements of synthetic dl alpha

tocopherol acetate.

2. Failure of implantation was observed after the eighth month in females without tocopherol supplements. With continuous tocopherol administration, the implantation rate was normal after one year. Single post-mating tocopherol supplements increased the implantation rate in older rats significantly. The requirements necessary for successful implantation in older rats vary widely and increase steeply with age.

3. Infections of the uterus and the tubes were frequently observed in the deficient rats, but not in those on the complete diet. Presence of infection does not explain the higher implantation rate after single post-mating tocopherol doses.

4. The changes leading to the failure of implantation are prevented, but once present, only partly counteracted by alpha tocopherol acetate.

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Amputation of the Canine Atrial Appendages.*

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Thrombus formation in the left atrial appendage and embolization therefrom are frequent sequelae of rheumatic mitral stenosis. Surgical excision of the thrombus-containing appendage suggests itself as a possible therapeutic approach to this problem. As a preliminary step experiments were performed to determine the effect of amputation of one or both atrial appendages in normal dogs.

Methods. The surgical procedures under anaesthesia, consisted essentially of exposure of the heart through an incision in the left fifth or sixth intercostal space, pericardiotomy, presentation of the atrial appendage with an Allis clamp, isolation of the appendage from the atrium with a right angle clamp or curved hemostat, ligation of the base of the appendage proximal to the clamp and finally amputation of the appendage. Excision of the left atrial appendage was accomplished with the animal in the left lateral position.

Electrocardiograms were obtained pre-operatively and at varying intervals during the post-operative period. The animals were sacrificed and necropsy performed 4 to 12 weeks following surgery. Eight animals were used, 5 undergoing left, 2 right and one bilateral atrial appendectomy.

Results. Seven of the 8 dogs survived the operation. The first animal developed respiratory difficulty and died 15 minutes after closure of the chest. All the surviving animals made uneventful recoveries. Serial electrocardiograms failed to reveal abnormal atrial rhythms or evidence of atrial injury except in one dog which developed transient intra-atrial block. Necropsy revealed complete endothelialization at the site of the atrial scar. No subjacent mural thrombi were found. Pericardial and pleuro-pericardial adhesions occurred.

Conclusion. Excision of one or both atrial appendages is feasible in dogs.

* Aided by the Emil and Fanny Wedeles Fund for Cardiovascular Research.

Perirenal Hemorrhage in Ureter Ligated Dogs Receiving Phthalylsulfathiazole.

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Reports of toxic manifestations following the administration of succinylsulfathiazole or phthalylsulfathiazole are infrequent and are considered to be of little consequence. In the course of some experiments on the treatment of experimental uremia¹ it was noted that the combination of ureter ligation in dogs and the oral administration of phthalylsulfathiazole resulted in hemorrhage beneath the fascial sheath covering the perirenal fat. Further studies were done in an attempt to discover the cause of this bleeding. The results of these studies are reported here.

Procedure. The animals used were mongrel dogs weighing 6 to 14 kg. Six test animals received 0.5 to 0.75 g/kg/day of phthalylsulfathiazole in oral divided doses for 3-6 days before the ureters were ligated and thereafter until the death of the animal. In addition, the ureters of 6 control dogs were ligated; the operation in both cases was done under ether anesthesia with morphine-atropine premedication, except Dogs 6, 7, and 9 which were anesthetized with pentobarbital sodium (30 mg/kg). The ureters were located and doubly ligated with silk about one cm from their entrance into the bladder. The animals were observed until their death.

In 4 of the test animals, (No. 8, 10, 11 and 12) blood sulfathiazole levels* were determined within 24 hours prior to death according to the method of Bratton and Marshall.² Serial blood phenol determinations, according

to the method of Bernhart and Schneider,³ were done in some of the test and control animals to follow the course of the uremia. In 3 dogs receiving the phthalylsulfathiazole (No. 10, 11 and 12) daily, pre- and post-operative prothrombin times according to the method of Quick,⁴ red blood cell and platelet counts, and bleeding, clotting, and clot retraction times were done. Autopsies were done on all animals as soon after death as possible.

Results. All animals in which there was evidence that urine flow had been reestablished were omitted from the series. The significant pathologic findings are recorded in Table I. Of the 6 ureter ligated animals receiving phthalylsulfathiazole, 4 had gross hemorrhage beneath the perirenal sheath (Fig. 1), and of these, 3 had free blood in the abdomen. Of the remaining two test animals, one had a marked increase in vascularity of the perirenal sheath with a small amount of perirenal hemorrhage, and the other animal had a moderate increase in perirenal vascularity without perirenal hemorrhage.

Four of the control animals, without sulfonamide administration, showed moderately increased perirenal vascularity at autopsy, but none showed perirenal sheath hemorrhage. One had a small amount of old hemolyzed blood in the abdomen, but since this could not be attributed to a perirenal lesion it was thought to be due to the operative procedure.

Of the 3 test animals in which hematologic studies were done, one showed perirenal sheath hemorrhage and free blood in the abdomen, one showed a marked increase in perirenal vascularity without hemorrhage, and one showed a moderately increased perirenal vascularity. None of these animals showed any

¹ Wallace, S. L., Little, J. M., and Bobb, J. R. R., in preparation.

* We are indebted to the staff of the Clinical Chemistry Laboratory for the sulfathiazole determinations reported in this paper.

² Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

³ Bernhart, F. W., and Schneider, R. W., *Am. J. Med. Sci.*, 1943, **205**, 636.

⁴ Kracke, R. R., *Diseases of the Blood*, 2nd Ed., J. B. Lippincott Co., Philadelphia, 1941.

TABLE I

Dog No.	Increased vascularity of perirenal sheath	Pathologic findings			
		Hemorrhage beneath perirenal sheath	Free blood in abdomen	Capsular hemorrhage	Kidney substance hemorrhage
Control					
1	Moderate	None	None	None	None
2	"	"	"	"	"
3	"	"	"	"	"
4	None	"	Yes*	"	"
5	Moderate	"	None	"	"
6	None	"	"	"	"
Test					
7	**	Yes	None	None	None
8	**	"	Yes	"	"
9	**	"	"	"	"
10	**	"	"	"	"
11	Marked	Small amt	None	"	"
12	Moderate	None	"	Punctate	"

* This animal had 50-75 cc of old, hemolyzed blood in its abdomen with no observable origin. There were no changes in the perirenal tissues.

** Because of the perirenal sheath hemorrhage in these animals, it was not possible to determine if they had increased vascularity of the sheath.

hematologic abnormalities either pre- or post-operatively. In addition, none of the test animals during life or at autopsy showed any evidence of hemorrhage in any other tissue that was not also seen in the control animals. All animals showed punctate subendocardial hemorrhage on the valves and ventricular wall.

The average post-operative survival time of the animals receiving phthalylsulfathiazole was 79 hours with a range of from 54 to 118 hours. The average post-operative survival time of the control animals was 72 hours, with a range of from 37 to 109 hours. The post-operative course in the two groups was essentially the same, except that some of the animals subsequently showing free blood in the abdomen died more acutely, presumably of shock.

There was no significant difference in the blood phenol levels before death in the two series of animals. The blood sulfathiazole levels taken before death averaged 2.9 mg %, with a range of 2.5 to 3.4 mg %. The combined form of sulfathiazole averaged 0.1 mg %.

Discussion. The principal difference between the control and test groups of animals was the presence of hemorrhage beneath the perirenal sheath and free blood in the peri-

toneal cavity in the test group. We have found in other experiments (unpublished) that such hemorrhage does not occur with phthalylsulfathiazole administration to normal dogs, to dogs subjected to bilateral nephrectomy, or to dogs with ureter ligation in which the urine flow is reestablished. Therefore the responsible factors involved in producing the hemorrhage are the administration of phthalylsulfathiazole and the presence of ureteral obstruction with intact kidneys. Neither factor alone is sufficient.

The hemorrhage could be due either to hematologic changes or to vascular changes or to a combination of the two. The complete absence of abnormal hematologic findings suggests that vascular abnormalities are responsible. It appears that the increased perirenal vascularity accompanying ureteral ligation is a predisposing factor for hemorrhage upon phthalylsulfathiazole administration, but the nature of the highly localized vascular changes is unknown.

Winternitz and Katzenstein,⁵ in a review of the experimental pathology of ureteral obstruction, report that at autopsy the kidney capsular vessels were prominent and congested and that the surface of the kidney in many

⁵ Winternitz, M. C., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **13**, 15.



Fig. 1.
Hemorrhage beneath the fascial sheath covering the perirenal fat in ureter ligated dogs which received phthalylsulfathiazole.

cases was covered with a dark red friable exudate which usually extended into the surrounding perirenal adipose tissue. In none of their animals was perirenal hemorrhage reported. There was also no mention of such hemorrhage after ureteral ligation in the report of Harrison and Mason.⁶

Mattis, Benson and Koelle⁷ reported that in one experiment they gave 1.0 g/kg of phthalylsulfathiazole intraperitoneally daily for 6 days to a monkey. The animal died on the sixth day with severe abdominal pain, hematuria, crystalluria, nausea, vomiting, weakness, and diarrhea. Autopsy showed extensive crystalline deposits in the renal parenchyma and pelvis and a marked recent bloody exudation in the peritoneal cavity. No mention was made of perirenal hemorrhage, but it is possible that the bloody exudate found represented such a hemorrhage since the animal had renal obstruction due to sulfonamide crystals. With this possible exception, the experimental^{7,8} and clinical⁹⁻¹³ administration of phthalylsulfathiazole has not been shown to cause bleeding in any

tissue. Poth⁹ quotes Allen to the effect that one-fifth of his patients with carcinoma of the colon had increased bleeding on treatment with succinylsulfathiazole, but he reported that this did not occur following phthalylsulfathiazole.

The observations reported here suggest that the clinical administration of phthalylsulfathiazole should be avoided in the presence of diminished renal function due to ureteral obstruction.

Summary. The ureters were ligated in 6 dogs, and phthalylsulfathiazole was administered orally in doses of 0.5 to 0.75 g/kg/day for 3-6 days pre-operatively and post-operatively until death. Two of these animals showed at autopsy an increased vascularity of the fascial sheath covering the perirenal fat, 5 animals showed perirenal sheath hemorrhage, and 3 animals had fresh free blood in the abdominal cavity.

The ureters were ligated in 6 control dogs, and they received no phthalylsulfathiazole. Four of these animals showed at autopsy an increased vascularity of the perirenal sheath. None of the animals showed perirenal hemorrhage.

It was found that both ureter ligation and phthalylsulfathiazole administration are necessary for production of the perirenal lesions. It appears that the perirenal hemorrhage is due to vascular changes since no abnormalities in prothrombin time, bleeding, clotting, and clot retraction time, red blood cell counts or platelet counts were demonstrated. The average total blood sulfathiazole concentration at the time of death in the test animals was 2.9 mg %.

⁶ Harrison, T. R., and Mason, M. F., *Medicine*, 1937, **16**, 1.

⁷ Mattis, P. A., Benson, W. M., and Koelle, E. S., *J. Pharm. and Exp. Therap.*, 1944, **81**, 116.

⁸ Poth, E. J., and Ross, C. A., *Texas Rep. Biol. and Med.*, 1943, **1**, 345.

⁹ Poth, E. J., *Surgery*, 1945, **17**, 773.

¹⁰ Poth, E. J., *Texas Rep. Biol. and Med.*, 1946, **4**, 68.

¹¹ Streicher, M. H., *J. A. M. A.*, 1945, **129**, 1080.

¹² Angelo, G., *Am. J. Surg.*, 1945, **70**, 354.

¹³ Bargen, J. A., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 85.

Complement Fixation Studies in Experimental Histoplasmosis.

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Chiefly because of the absence of a specific, potent antigen, no satisfactory complement-fixation test has been recorded for histoplasmosis. Van Pernis, Benson and Holinger¹ conducted complement-fixation tests on a human patient with histoplasmosis, but obtained negative results. The antigens they used were (a) the undiluted filtrate from a dextrose broth culture medium inoculated with *H. capsulatum* and (b) the acetone precipitate redissolved in saline.

However, antigens have been prepared and successfully used for complement-fixation tests in other mycotic diseases. For example, Martin^{2,3} employed antigens derived from the yeastlike phases of *B. dermatitidis* and *Candida sp.* for the fixation of complement in the presence of homologous antisera, whereas Martin,⁴ Fonseca⁵ and Smith⁶ derived their complement-fixation antigens from the mycelial phase in their studies on chromoblastomycosis, South American blastomycosis and coccidioidomycosis respectively.

Since the causative organism of histoplasmosis appears in the yeast-like form in infected animals and human beings, an antigen derived from this phase might be expected to produce the most satisfactory serologic tests for this disease. After the yeastlike form of *H. capsulatum* was successfully grown in a semi-fluid medium in large quantities,⁷ serologic studies were initiated. The purposes of these investigations were (1) to determine the binding power and specificity

of the yeastlike phase of *H. capsulatum* as a complement-fixation antigen, and (2) to obtain information on the time of appearance, the maximum titer and the persistence of complement-fixation antibodies in animals infected with histoplasmosis. In this paper the results of these investigations are presented.

Materials and Methods. a. *Complement-fixation antigens.* The yeastlike phase of *H. capsulatum* was grown at 37°C in semi-fluid medium⁷ for 3 weeks, centrifuged, washed in Tyrode's solution at pH 7.0, and killed by 0.5% formalin. The cells were then centrifuged at 32-36°F, washed twice in Tyrode's solution, and stored at 2-5°C. The antigen employed in the complement-fixation tests consisted of whole washed formalin-killed cells of the yeastlike phase, and will henceforth be referred to as "YP antigen." Although all lots had approximately equal potency, one lot of YP antigen (derived from strain 6521) was used in the following experiments. The antigenic titer was 1:256 against rabbit immune serum and the anti-complementary titer was 1:4-1:8. This antigen exhibited similar binding power in the presence of antisera against the homologous strain and against five other strains of *H. capsulatum*.

Histoplasmin is the broth filtrate from the growth of the mycelium of *H. capsulatum* on a modified Long's medium.⁸ The fungus was grown at room temperature in the dark, with samples removed periodically for assay. During approximately the first 3 weeks of growth, the filtrate had no binding power in the presence of homologous antiserum. However, for the next 20-30 days a marked rise in binding power of the filtrate appeared (Fig. 1). Thereafter the titer remained at a fairly con-

¹ Van Pernis, P. A., Benson, M. E., and Holinger, P. H., *J. A. M. A.*, 1941, **117**, 436.

² Martin, D. S., *J. Infect. Dis.*, 1935, **57**, 291.

³ Martin, D. S., *Am. J. Trop. Med.*, 1942, **22**, 295.

⁴ Martin, D. S., *Am. J. Trop. Med.*, 1938, **18**, 421.

⁵ da Fonseca, O., *An. brasil. de dermat. e sifil.*, 1939, **14**, 112.

⁶ Smith, C. E., personal communication.

⁷ Salvin, S. B., *J. Bact.*, 1947, **54**, 655.

⁸ Emmons, C. W., Olson, B. J., and Eldridge, W. W., *Pub. Health Rep.*, 1945, **60**, 1383.

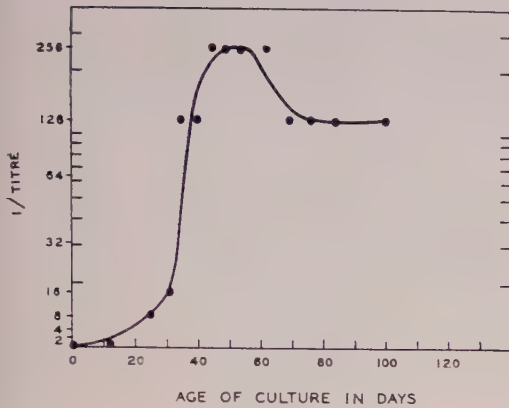


FIG. 1.

Development of binding power of histoplasmin from strain 6510 of *Histoplasma capsulatum* against homologous antiserum.

stant level, although there was a slow but distinct increase in the anticomplementary action as the culture aged, especially after the first 90 days of growth. For the experiments in which histoplasmin was used, a 60-day-old filtrate of strain 6510 was employed as the antigen.

b. *Complement-fixation test.* The method was that employed by Bengston.⁹ Complement was titrated in the presence of antigen each day tests were conducted and antigens were tested whenever new lots were employed. Four units of antigen, 2 of complement, and $1\frac{1}{2}$ of amboceptor were used throughout the studies.

c. *Strains of fungi.* The fungi were from the collection in this laboratory. The yeastlike phase on which most of the studies were conducted was from a strain (No. 6521) from a naturally-infected dog.¹⁰ The histoplasmin employed was produced by a culture of strain 6510.¹¹ Blastomycin was obtained from a broth culture of strain 6014 of *B. dermatitidis* isolated by Conant; and the coccidioidin, partly from a broth culture of strain 6210 of *C. immitis* isolated by Emmons, and partly supplied by C. E. Smith.

⁹ Bengston, I. A., *Pub. Health Rep.*, 1944, **59**, 402.

¹⁰ Olson, B. J., Bell, J. A., and Emmons, C. W., *Am. J. Pub. Health*, 1947, **37**, 441.

¹¹ McLeod, J. H., Emmons, C. W., Ross, S., and Burke, F. G., *J. Pediat.*, 1946, **28**, 275.

d. *Animals.* The experimental animals were adult albino rabbits, which were bled and their sera tested for complement-fixation antibodies in all cases with the appropriate antigens before inoculation. Among 228 animals tested, only 2 rabbits had sera that fixed complement in the presence of histoplasmin, and none in the presence of YP antigen. What caused these two rabbit sera to fix complement in serum dilutions of 1:8 and 1:16 was not learned, since no gross abnormalities were discernible. However, the two were discarded.

Experiments and Results. a. *Production of complement-fixation antibodies.* Fifteen rabbits were inoculated intravenously with a suspension of the live yeastlike phase of strain 6521 of *H. capsulatum*. They were bled from the ear veins periodically, and their serum assayed for complement-fixing antibodies with YP antigen.

For about 10 days after inoculation, no antibodies were demonstrable (Fig. 2). The antibody titer then began to increase sharply until by about the 30th day the antibody titer reached 1:512 for most of the animals. All rabbits developed antiserum that fixed complement in the presence of YP antigen. A rapid decrease in titer occurred for the 20 ensuing days, after which the titer remained more or less constant until the experiment was terminated 170 days after inoculation.*

When these same antisera were tested with histoplasmin as the antigen, much lower antibody titers were obtained (Fig. 2), the highest being 1:128 in comparison with the 1:512 with YP antigen. In addition, 7 of the 15 animals showed no positive titers at any time, in contrast to the positive titers demonstrated in all the animals with the YP antigen. With histoplasmin as the complement-fixation antigen, the maximum titer was attained during the third week after inoculation, after which the antibody titer decreased progressively until the eighth week, when no antibodies could be demonstrated.

b. *Reactions of H. capsulatum antigens with other fungal antisera.* (a) *Coccidioidomycosis.* YP antigen and histoplasmin were

* Not all the rabbits were bled on each "bleeding day."

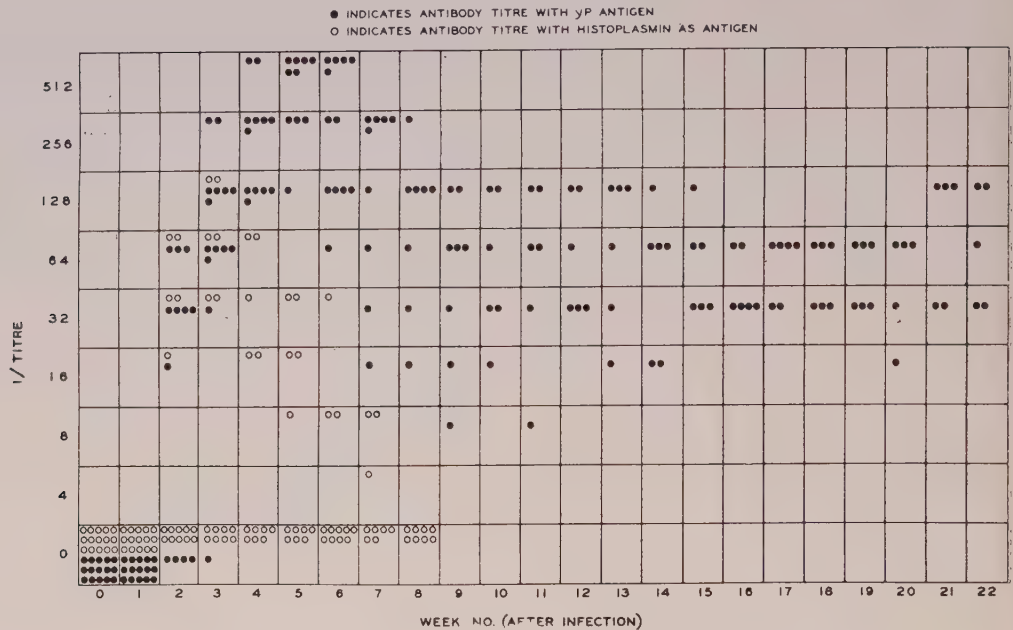


FIG. 2.

Development of antibody titer of experimentally infected rabbits in the presence of (a) YP antigen and (b) histoplasmin.

Note: The decrease in the number of readings per week is due to the deaths of an increasing number of animals.

studied in complement-fixation tests with antisera from 8 rabbits experimentally infected with *C. immitis* and from 10 human patients with localized or disseminated coccidioidomycosis.[†] All the rabbits infected with *C. immitis* developed potent complement-fixation antibodies in the presence of the homologous antigen. However, at no time did YP antigen fix complement in the presence of coccidioidomycosis antibodies, although histoplasmin did, both with the rabbit and human antisera.

(b) *Blastomycosis*. Eight rabbits, inoculated intravenously with the living yeastlike phase of *B. dermatitidis*, were bled periodically and their antisera analyzed for the presence of complement-fixation antibodies with blastomycin as antigen. Antibodies were demonstrated about 20 days after infection, and remained until the ninth week. Throughout this period of demonstrable antibody with the homologous antigen, YP antigen produced no complement-fixation with the blastomy-

cosis antisera at any time. However, histoplasmin showed reactions with these heterologous antisera at the low titers of 1:8 and less.

(c) *Moniliasis*. Four rabbits were immunized by daily intravenous injections of formalin-killed cells of the budding phase of *Candida albicans*. The resulting antisera, active with the homologous antigen, were analyzed for complement-fixing properties in the presence of histoplasmin and YP antigen. Histoplasmin produced complement-fixation in antiserum dilutions as high as 1:8, whereas YP antigen showed no positive reactions whatsoever.

Discussion. Antigens from the yeastlike phase of *H. capsulatum* show promise as agents for determining the presence of complement-fixation antibodies, since these antigens have specificity, good binding power, and low anticomplementary titers with experimentally produced rabbit antisera. When histoplasmin was used as antigen, the titers were lower, of shorter duration, and less specific than with the YP antigen. Also of

[†] Thanks are expressed to Dr. C. E. Smith for providing these antisera.

interest was the persistence of a positive titer in the presence of YP antigen in the sera of rabbits with histoplasmosis after gross external symptoms of the disease had disappeared and blood cultures were no longer positive.

During the course of these studies on laboratory animals, two human patients appeared from whom *H. capsulatum* was isolated.[†] Their sera fixed complement in the presence

[†] Thanks are expressed to Drs. F. C. Kelly and J. Wells for making these sera available.

of YP antigen in dilutions of 1:32 and 1:128. More than 30 "normal" persons from whom *H. capsulatum* was not isolated had no complement-fixing antibodies in the presence of YP antigen.

Summary. An antigen from the yeastlike phase of *Histoplasma capsulatum* fixed complement in the presence of antisera from experimentally infected rabbits from the 2nd to the 23rd weeks following infection. In contrast to histoplasmin, this antigen did not react with several other fungal antisera.

16087

Sulfacin. Bacterial Spectrum, Toxicity and Therapeutic Studies.*

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A new antibiotic, sulfacin, has been described by Junowicz-Kocholaty, Kocholaty, and Kelner.¹ A description of the actinomycetes, the method for growing the organism, and the purification and chemical properties of the antibiotic substance were given by the above authors. It is the purpose of this publication to present results of studies on the bacterial spectrum and the toxicity and therapeutic action in mice.

Experimental. The sterile crude culture filtrate of Actinomycetes, R-30, was employed for determining the bacterial spectrum. In general, the *in vitro* antibacterial tests were carried out at the time similar tests were being made with actinorubin and lavendulin employing Bacto-nutrient broth adjusted to pH 7.3 and by the technic described previously by Kelner and Morton.² Since sulfacin is more inhibitory for gram-positive than for

gram-negative organisms, it was standardized against *Staphylococcus aureus*, P210. The smallest amount of sulfacin per ml of Bacto-nutrient broth, pH 7.3 which prevented growth of *S. aureus*, under the conditions of the test, was designated as one dilution unit.

The crude culture filtrate of Actinomycetes, R30, was titrated against *S. aureus*, P210, in Bacto-nutrient broth, pH 7.3, and in the same medium to which had been added 0.5% NaCl. In each medium the activity titrated to the same tube, representing 6,400 dilution units. Thus the antibacterial action of sulfacin is not appreciably reduced in the presence of 0.5% NaCl. The presence of 10% sterile, defibrinated, normal horse blood in the Bacto-nutrient broth with salt necessitated 10 times more sulfacin to inhibit *S. aureus*.

There was received on June 20, 1946, from Dr. Junowicz-Kocholaty about 60 mg of recrystallized sulfacin which had been dried over CaCl₂. By the agar streak technic of assay it was estimated that 0.0254 μ g of this material equalled one unit. The material was put in solution as follows: To 10.43 mg of sulfacin was added one ml of chloroform and warmed to 45°C until dissolved. One ml of

* This work has been supported by a grant from the Smith, Kline, and French Laboratories, Philadelphia, Pa.

¹ Junowicz-Kocholaty, R., Kocholaty, W., and Kelner, A., *J. Biol. Chem.*, 1947, **168**, 765.

² Kelner, A., and Morton, H. E., *J. Bact.*, 1947, **53**, 695.

TABLE I.
Results of *in vitro* Antibacterial Tests with Sulfactin.

Organism	Strain	Amt of sulfactin/ml of medium inhibiting the strain
<i>Aerobacter aerogenes</i>	P41	>32
<i>Alcaligenes faecalis</i>	P61	>32
<i>Brucella abortus</i> *	P62, P18A	>32
" <i>melitensis</i> *	P80	>32
" <i>swis</i> *	P64	>32
<i>Chromobacterium violaceum</i>	P104	>32
<i>Eberthella typhosa</i>	P11, P115	>32
<i>Escherichia coli</i>	P216	>32
" <i>communior</i>	P218	>32
" <i>neapolitania</i>	P161	>32
<i>Klebsiella pneumoniae</i>	ATCC No. 8050	>32
<i>Mycobacterium smegmatis</i>	P49	>32
<i>Neisseria catarrhalis</i>	P66	>32
<i>Proteus vulgaris</i>	P98, P188	>32
<i>Pseudomonas aeruginosa</i>	P2, P186A	>32
<i>Salmonella enteritidis</i>	P51	>32
" <i>paratyphi</i>	P198A	>32
" <i>schottmuelleri</i>	P35	>32
<i>Sarcina lutea</i>	P6	>32
<i>Serratia marcescens</i>	P4	>32
<i>Shigella dysenteriae</i>	P52A	>32
" <i>paradysenteriae</i> , Flexner	P21	>32
<i>Streptococcus faecalis</i> †	P122	>32
<i>Trichophyton interdigitale</i>		>32
<i>Vibrio comma</i>	P215	>32
Alpha streptococcus†	P26	32
Gamma streptococcus†	P169	32
<i>Bacillus anthracis</i>	P119	16
" <i>cereus</i>	P109	16
" <i>mycoides</i>	P34A, P58A	16
" <i>anthracis</i>	P60	8
<i>Gaffkya tetragena</i>	P140A	8
<i>Bacillus subtilis</i>	P219	4
<i>Streptococcus pyogenes</i> †	P24	4
<i>Bacillus circulans</i>		2
" <i>megatherium</i> .	P97	2
" <i>mycoides</i>	P33A	2
" <i>subtilis</i>	P23A	1
<i>Diplococcus pneumoniae</i> , type 1†	P27	1
<i>Staphylococcus aureus</i>	P210	1
<i>Bacillus mesentericus</i>	P112	0.5
" <i>mycoides</i>	P156	0.5
" <i>subtilis</i>	P7	0.5
<i>Diplococcus pneumoniae</i> , type 3†	P29	0.5
<i>Gaffkya tetragena</i>	P43	0.5
<i>Neisseria sicca</i>	P141A	0.5
<i>Micrococcus aurantiacus</i>	P103	0.25
" <i>lysodekticus</i>		0.25
" <i>roseus</i>	P120B	0.125
<i>Corynebacterium xerose</i>	P82	0.06
" <i>diphtheriae</i>	P1	0.03

* The tests with *Brucella* were made in Bacto-tryptose broth.

† Tests with *Streptococci* and *Diplococci* were carried out in Bacto-nutrient broth, pH 7.3, to which had been added 0.5% NaCl and 5% sterile, normal defibrinated horse blood.

95% ethyl alcohol was added then distilled water to make 10 ml. When assayed by the technic employing broth, the material appeared considerably more active than when assayed by the agar streak technic. In broth 0.0048 μ g of sulfactin per ml inhibited *S.*

aureus. It was found that a concentration of 0.0097 μ g per ml of Bacto-nutrient broth to which had been added 0.5% NaCl and 5% sterile, normal, defibrinated horse blood prevented visible growth of *Diplococcus pneumoniae*, type 1, P28 for 48 hours at 37°C.

TABLE II.
The Toxicity of Sulfactin for White Mice by Intraperitoneal Injection.

Dose of sulfactin	No. of mice injected	No. of mice living	No. of mice dying
0.1 mg	5	5	0
0.5	5	5	0
1.0	5	4	1*
5.0	4	0	4†

* The animal was found dead on the morning of the 8th day. A gram-negative rod was isolated from the heart's blood. The tissues were too badly autolyzed for a critical pathological examination.

† All animals died within 3.5 hours after injection. All animals showed a reaction within 2 minutes after injection; one mouse had violent tremors. It was not entirely satisfactory working with concentrations of 5 mg of sulfactin per ml of water because of the insolubility of the product at that concentration. The sulfactin was put into solution with chloroform at 45-50°C, distilled water added, and most of the chloroform evaporated by bubbling a small stream of sterile air through the liquid. This resulted in a heavy white suspension, relatively free of chloroform vapor. It is not certain that the early deaths of the mice receiving 1 ml of the suspension containing 5 mg of sulfactin can be attributed to the pharmacologic action of the drug. Nothing abnormal could be detected by gross examination, except, perhaps, that the liver and kidneys appeared a bit pale. Microscopic examination revealed that the capillaries in the lungs contained an excessive number of polymorphonuclear leukocytes. The pancreas, at the periphery of the lobes in 3 of the 4 mice, showed well marked degeneration of the outer zone of acini. Large numbers of fragmented nuclei were found in the "reaction centers" of the thymus and lymphoid follicles of the spleen. Other tissues appeared essentially normal. Pathological studies did not permit an opinion of the mechanism of death of the animals.

Because of the high virulence of our strain, this organism was selected for therapeutic studies.

The toxicity of sulfactin was determined by intraperitoneal injection into white mice weighing 17 to 20 g each. The results are summarized in Table I. By applying the formula of Reed and Muench³ to the results, the LD₅₀ is calculated to be about 2.75 mg. The necessity for employing the LD₅₀ is set forth in the footnotes to Table I.

The mice which survived the injections of sulfactin were sacrificed for pathological studies. Two mice which received one mg each of sulfactin were sacrificed 14 days after the injection of the drug. Possible slight enlargement of the liver was the only gross pathological change detectable. These changes were in no ways as extensive as those observed in mice following the injection of 0.75 mg of actinorubin.⁴ Two mice which received 0.5 mg each of sulfactin were sacrificed 14 days after the injection and a possible enlargement of the liver was the only gross pathological change observable. Two mice which received 0.1 mg each of sulfactin

were sacrificed 14 days after the injection and no gross pathological changes were detectable.

Twenty-one days post-injection 2 mice which had received one mg, 2 mice which had received 0.5 mg and 2 mice which had received 0.1 mg sulfactin each were sacrificed. Nothing abnormal could be observed microscopically. None of the organs of the mice sacrificed 14 days and 21 days post-injection showed anything abnormal by microscopic examination. We are indebted to Dr. Herbert Ratcliffe for the pathological studies on these animals.

For a preliminary test of the therapeutic potentialities of sulfactin it was tested against *D. pneumoniae*, type I in mice. Serial dilutions of a 24-hour-old blood broth culture of the test organism were made in nutrient broth. One ml portions of the various dilutions of the test organism were inoculated intraperitoneally into mice to determine its pathogenicity, and one ml of each dilution was made into a poured blood agar plate to estimate the number of organisms constituting a lethal dose. Those mice receiving sulfactin received it intraperitoneally within a few seconds after the injection of the culture. The results are summarized in Table II. The results of the protection test made on July 2nd appeared so promising that a second ex-

³ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

⁴ Morton, H. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **64**, 327.

TABLE III.
 Ability of Sulfactin to Protect Mice Infected with *Diplococcus pneumoniae*, Type 1.

Diln. of culture, 1 ml/mouse	No. of organisms dose*	No. of mice inj.	Amt of sulfactin inj.	No. of mice living	No. of mice dying
Experiment of July 2, 1946.					
10-5	∞	5	0	0	5
10-6	284	5	0	0	5
10-7	28	5	0	0	5
10-8	5	5	0	0	5†
10-9	0	5	0	3	2†
10-5	1000 M.L.D.	5	0.1 μ g	0	5†
10-5	" "	5	0.5 "	0	5†
10-5	" "	10	1.0 "	5	5†
10-5	" "	5	2.0 "	1	4†
10-5	" "	5	10.0 "	5	0
Experiment of July, 10, 1946.					
10-5	∞	5	0	0	5
10-6	>300	5	0	0	5†
10-7	51	5	0	0	5
10-8	8	5	0	1	4†
10-9	1	5	0	4	1†
10-10	1	5	0	5	0
10-5	<1000 M.L.D.	10	0.5 μ g	0	10†
10-5	" "	10	1.0 "	0	10†
10-5	" "	10	5.0 "	4	6†
10-5	" "	10	10.0 "	6	4†

* No. of organisms estimated by inoculating 1 ml, the same amount as was injected into each mouse, into a poured blood agar plate.

† *D. pneumoniae* isolated from the heart's blood at autopsy.

‡ Three mice died between the 2nd and 3rd day after injection and pneumococci were isolated from the heart's blood of each. One mouse was found dead in the cage during the 6th day with its nose chewed. The heart's blood culture was sterile, so it is doubtful that it died of pneumococcal infection.

periment was made eight days later. In the first experiment the amount of sulfactin which protected 50 per cent of the mice against 1,000 minimum lethal doses of culture was in the order of 1 or 2 μ g. This compares quite favorably with the LD₅₀ toxic dose of about 2,750 μ g. In the second experiment the challenging dose of culture may have been slightly less than 1000 M.L.D. The sulfactin was another lot prepared from the original purified material. The amount of sulfactin which protected against nearly 1000 M.L.D. of culture was of the order of 7.5 μ g. This is still a quite favorable comparison to the toxic dose. There was not sufficient material avail-

able for further toxicity and therapeutic studies.

Studies on drugfastness and comparison with penicillin in this respect have been made and will be reported later.

Summary. Sulfactin is active primarily against gram-positive microorganisms. The LD₅₀ for mice of 17-20 g by intraperitoneal injection is about 2,750 μ g. The amount of sulfactin required to protect mice against the intraperitoneal injection of approximately 1000 minimum lethal doses of *Diplococcus pneumoniae*, type 1, is of the order of 1 to 7.5 μ g.

Experimental Type Transformation of *Shigella paradysenteriae* (Flexner).

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Griffith and Dawson observed type transformation of pneumococci *in vivo* and *in vitro* induced by dead pneumococci or extracts of the transforming type. The transforming principle was shown by Avery and his collaborators to be a desoxyribonucleic acid (for references see¹). Recently, Boivin^{2,3,4} reported transformation of an *E. coli* strain induced by a filtrate strain of a different type. Again the inducing agent was identified as a desoxyribonucleic acid. The theoretical aspects of these observations have been discussed.⁵

Considerable information obtained in recent years on the antigenic properties of *Shigellae*, particularly *Shigella paradysenteriae* (Flexner)^{6,7} provide a basis for an investigation of type transformation of *Shigellae*. During the last 2 years several attempts were made to obtain evidence of this kind. The first results were disappointing, but information worthwhile mentioning here was obtained: we found it difficult to produce true R variants of Flexner bacilli on a number of fluid and solid media, including synthetic ones⁸ providing only minimal requirements for growth. Cultivation in homologous immune serum in concentrations up to 10% was also not conducive to the appearance of R variants. Bacteria grown in broth containing up to 10% of homologous or heterologous

immune serum or in normal serum gave no indications of changes in their antigenic behavior. Also addition of formol-killed bacteria to nutrient broth of a heterologous type did not influence the cultural and serological properties of *Shigellae*. However, in the light of experiences reported below it cannot be excluded that the number of experiments with this technique was insufficient to disprove the possibility of obtaining type transformation with killed organisms as inducing agent.

However, we have been able to secure evidence of type transformation in Flexner bacilli with extracts similar to those employed by Boivin.

Technique. Cultures of *Sh. paradysenteriae* were grown in beef heart broth (without dextrose, pH 7.4) at 37°C for approximately 18 hours. Ten drops of toluene were added to each 40 ml of culture, which was kept at room temperature for 4 hours under repeated shaking. The bacteria were separated by centrifugation in an angle centrifuge placed in a chillroom ($\pm 4^\circ\text{C}$). The supernatant fluids of two 40 ml tubes were pooled and sent through a filter candle of medium porosity. The filtrates were stored in the chillroom and distributed in 3 ml volumes in small test tubes.

The filtrates were inoculated from cultures grown 18 hours at 37°C on beef heart agar slants. After incubation at 37°C overnight platings on beef heart agar were made. They were repeated one or several days later as desired. Sterility tests accompanied each step of the procedure, and an uninoculated tube was incubated and plated out together with the inoculated filtrates.

Strains from our collection whose stability in serological and cultural properties was known for at least one year were employed. They were examined anew before entering the experiments.

¹ McCarty, M., *Bact. Rev.*, 1946, **10**, 63.

² Boivin, A., Delaunay, A., Vendrely, R., and Lehault, Y., *Experientia*, 1945, **1**, 334.

³ Boivin, A., Delaunay, A., Vendrely, R., and Lehault, Y., *Experientia*, 1946, **2**, 139.

⁴ Boivin, A., and Vendrely, R., *Helvet. chim. acta*, 1946, **5**, 1338.

⁵ Luria, S. E., *Bact. Rev.*, 1947, **11**, 1.

⁶ Weil, A. J., Black, J., and Farsetta, K., *J. Immunol.*, 1944, **49**, 321 and 341.

⁷ Weil, A. J., *J. Immunol.*, 1947, **55**, 363.

⁸ Weil, A. J., and Black, J., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 24.

Colonies isolated from platings were examined by the slide agglutination technique with absorbed sera.^{6,7} If mutants were obtained, the colonies and, at the same time, non-mutated colonies were secured on agar slants and compared in test tube agglutinations and in cultural tests with the behavior of the parent strain and that of the strain from which the filtrate was derived ("inducing strain"). The following cultural methods were used in these comparative tests: beef heart agar for the observation of growth, colony form and microscopy, beef heart broth incubated both at room temperature and at 37°C, from which hanging drops were examined, appropriate media for tests of indol formation and of reduction of trimethylamine oxide, the decomposition of urea, the liquefaction of gelatin, and formation of acid and gas from dextrose, lactose, mannitol, maltose, dulcitol, rhamnose, arabinose, and salicin.

Filtrates were usually inoculated in groups of 6 with the corresponding strains, thus adding an additional safeguard by cross check. Altogether 38 filtrates involving Types I, II, II.VII, III, V, VI, VII, VIII, IX, and XII of the Flexner bacillus* were inoculated with at least 6 strains of different types. In 225 tests unequivocal evidence of type transformation was obtained in 3 cases, namely a filtrate of Type I inoculated with a strain of Type II.VII, a filtrate from a strain Type II inoculated with a strain of Type V, and a filtrate of a different strain Type II inoculated with a strain Type XII. In each case platings showed colonies both of the parent type and of the type of the inducing filtrate. In the first case, of 16 colonies examined, 10 were of Type II.VII and 6 of Type I; in the second case, of 40 colonies, 12 were of Type V and 28 were of Type II; and in the third case, of 95 colonies 49 were of Type XII and 46 of Type II. These figures were obtained from a single plating in the first case, and from 3 platings made on different days in the second and third case.

The Type V strain of the second case of transformation differed from the inducing strain of Type II, in that the Type V strain

was indol positive whereas the Type II strain did not form indol. All colonies from the platings reacting serologically like the parent strain gave a positive indol reaction, whereas the mutated colonies had lost the ability to form indol. As it happened, the parent and the inducing strain differed in a second property namely, the Type V strain does not form acid from maltose, whereas the inducing Type II strain acidifies this carbohydrate within 24 hours. Both non-mutated and mutated colonies from the platings did not acidify maltose. We then have here a mutation of one biochemical property coupled with the transformation of the somatic antigen, whereas a second property remains unchanged, thus tagging the mutants according to their parentage.

In the 2 other cases of transformation, the parent and the inducing strain were indistinguishable in their cultural properties and therefore did not offer a chance to obtain additional data on the association of mutation of biological and serological properties.

The parent Type II.VII strain of the first mentioned case of transformation grows on agar in slightly more opaque colonies than those of the inducing Type I strain. In the cultures obtained from the filtrates, those of serological Type II.VII were more opaque than the mutant colonies of Type I. This is illustrated in Fig. 1 where the parent and the inducing strain and one non-mutated and one mutated culture were plated out on separate quadrants of an agar plate. The difference in opacity may conceivably be merely the visible expression of the change in antigens. But it is also possible that opacity depends on some physical property of the bacteria independent from the somatic antigen. There is at present no way to decide whether or not the differences in opacity indicate another associated mutation.

The mutant strains have now been under observations for 10, 8, and 2 months respectively. During this period they were repeatedly plated out and numerous single colonies were tested for evidence of reversion to the original type. No such evidence was obtained.

Hitherto we have been unable to reproduce

* For type designation see 6.



FIG. 1.

Upper right: parent strain (type II.VII).

Upper left: inducing strain (type I).

Lower left: subculture from non-mutated colony (type II.VII).

Lower right: subculture from mutated colony (type I).

Note: The apparent paleness of some of the upper left hand section is due to the circumstance that this is the point of maximal illumination.

at will the phenomenon described. That is, new filtrates made from the inducing strains did not evoke mutations from the strains with which we were successful the first time.

Discussion. In 3 out of 225 attempts evidence of induced type transformation of Flexner bacilli by culture filtrates was obtained. In the single case where the parent and the inducing strain were distinguishable in their cultural behavior, one such distinguishing property, namely that of indol formation, was coupled to the change in somatic antigen, whereas a second one, namely acid formation from maltose, was not correlated with the serological transformation. Our experience resembles that of Boivin^{2,3,4} in two important points. Induced transformation could be produced in relatively rare instances—a single one in Boivin's case and 3 in ours. Moreover, Boivin found a mutation in a bio-

chemical capacity (sucrose fermentation) related with the change in antigen just as we found the serological mutation associated with the property of forming indol.

In our experience, the type transformation was not visibly mediated by a transition into the R phase. The lack of visible evidence of the R phase preceding transformation may, but does not necessarily, indicate a difference in the mechanism of transformation of *Sh. paradysenteriae* and that of *E. coli* and pneumococcus. Further study of this point is clearly indicated.

We were less fortunate than Boivin in that we have not been able to reproduce transformation at will with a given filtrate and a given strain, while Boivin evidently could, as indicated by the fact that he could collect amounts of filtrates large enough to proceed with an isolation of the desoxyribonucleic

acid. Thus, the factors involved in the induction of type transformation of Flexner bacilli require further study both concerning the parent organisms and the properties of filtrates.

The demonstration of induced type transformation in *Shigellae* is not only of theoretical interest,⁵ but it also cannot fail to influence our viewpoints on problems of the

biology of the enteric flora. These have just been discussed at some length,⁷ and it may suffice here to recall this aspect of induced type transformation.

Summary. Induced type transformation of *Sh. paradysenteriae* (Flexner) has been obtained in 3 out of 225 experiments. The inducing principle is contained in filtrates of broth cultures.

16089

Ovarian Hyperemia in the Immature Rat as a Pregnancy Test in Equids.

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Early diagnosis of pregnancy in equids by means of a biological reaction was studied by Cole and Erway.¹ A new method has been introduced by Zondek and Sulman² based on the ovarian hyperemia in immature rats injected with serum gonadotrophin. The same reaction proposed by Salmon *et al.*,³ as a 6-hour test for human pregnancy, was challenged by Farris⁴ and accepted with restrictions by Bunde.⁵ Recently Zondek and Sulman⁶ have recommended it again as a 24-hr test for the diagnosis of equine pregnancy. This note deals with the evaluation of the new test which was employed as a preliminary step for the preparation of mare gonadotrophin.

Experimental. Blood samples from 38 mares and 4 female donkeys obtained between

42 and 138 days after natural insemination were tested. Two infantile female rats weighing 40-55 g were injected subcutaneously with 1 and 2 cc respectively of each serum and killed 24 hours later. The ovaries were inspected in daylight and the response considered positive when they were enlarged and their color matched that of the kidney or the spleen. Pale yellow and small sized ovaries were indications of a negative response. Occurrence of pregnancy was assumed when one or both of the injected rats showed a positive response. With this technique and using rats from the same colony, positive reaction was always obtained 24 hours after the subcutaneous injection of 10 I.U. of a standard preparation of equine gonadotrophin. Therefore, when a positive response with 2 cc of serum was obtained, a blood concentration of at least 2,500 I.U. of gonadotrophin per liter was assumed to be present. Of course this is not an exact quantitative measurement but only a routine approximation.

A careful record was kept of each animal until evidence of pregnancy was established either by obvious abdominal enlargement or by subsequent parturition.

Results. Table I gives the results of the test with the serum of 38 mares and 4 female donkeys and the number of days between the natural insemination and the taking of

* Fellow of the J. S. Guggenheim Memorial Foundation.

¹ Cole, H. H., and Erway, J., *Endocrinology*, 1941, **29**, 514.

² Zondek, B., and Sulman, F., *Nature*, 1945, **3932**, 302.

³ Salmon, U. J., *et al.*, *J. Clin. Endocrinology*, 1942, **2**, 167.

⁴ Farris, E. J., *Am. J. Obst. and Gyn.*, 1944, **48**, 200.

⁵ Bunde, C. A., *Am. J. Obst. and Gyn.*, 1947, **53**, 317.

⁶ Zondek, B., and Sulman, F., *Endocrinology*, 1947, **40**, 322.

TABLE I.
Individual Data and Results of the Pregnancy Test in Mares and Female Donkeys.

Case No.	Age (years)	Wt (kg)	Days after insemination	Hyperemia reaction	Remarks
A. Mares					
1	9	501	90	Positive	Parturition, 330 days pregnant
2	13	470	78	"	Not pregnant*
3	12	400	80	"	Parturition, 327 days pregnant
4	15	336	72	"	" 328 " "
5	13	415	88	"	" 341 " "
6	18	343	68	"	Not pregnant*
7			72	"	Pregnant
8			56	Negative	Not pregnant
9			42	"	" "
10			78	Positive	Parturition, 327 days pregnant
11	15	370	80	"	Pregnant
12	9	370	71	"	"
13	13	420	81	Negative	Not pregnant
14	6	430	83	"	" "
15	10	450	69	Positive	Pregnant
16		400	62	Negative	Not pregnant
17	16	400	62	"	" "
18	5	330	76	Positive	Pregnant
19	11	390	78	"	"
20			87	"	"
21	12	410	85	Negative	" †
22	16	460	76	"	Not pregnant
23	9	340	64	Positive	Pregnant
24			114	Negative	Not pregnant
25			138	Positive	Parturition, 350 days pregnant
26			138	"	" 310 " "
27			138	"	" 335 " "
28	11	473	74	"	Not pregnant*
29	4	300	71	"	Pregnant
30	6	450	69	"	"
31	7	490	75	Negative	" †
32	5	255	57	"	Not pregnant
33	6	345	70	Positive	Pregnant
34	5	305	73	"	"
35	6	408	63	Negative	" †
36	13		65	"	" †
37	9	390	71	"	Not pregnant
38	7	370	61	Positive	Pregnant
B. Female donkeys					
1			75	Negative	Not pregnant
2			128	"	" "
3			133	Positive	Parturition, 368 days pregnant
4			113	"	" 357 " "

* False-positive tests.

† False-negative tests.

the blood sample. The clinical condition of the animal agreed with the biological test (both positive and negative reactions) in 35 cases. It did not agree in 7 instances. Four mares definitely pregnant gave negative test 85, 75, 65 and 63 days after fertilization. On the other hand, 3 non-pregnant mares gave positive test 78, 74 and 68 days after insemination.

Bunde⁵ pointed out that in human pregnancy, by employing the same hyperemia test, there occurred only false-negative test, *i.e.*

negative reaction with urine of pregnant women. In equids, however, there occurred both types, namely negative test with serum of pregnant mares and positive test with serum of non-pregnant ones. The false-negative tests are difficult to explain for, according to the data of Day and Rowlands,⁷ the high circulating level of gonadotrophin in mares occurs between the second and the fourth month of

⁷ Day, F. T., and Rowlands, I. W., *J. Endocrinology*, 1940, **2**, 255.

pregnancy. It would be necessary to assume that in the 4 false-negative cases cited in Table I, the concentration of blood gonadotrophin, when the serum was tested, was less than 2,500 I.U. per liter, despite the fact that it was 63 to 85 days after definitely established fertilization. The false-positive tests, *i.e.* positive reaction with serum of non-pregnant mares, occurred in 11-, 13- and 18-year-old animals. It is possible that age may increase the blood level of gonadotrophin in equids more markedly than in postmenopausal women, but it is unknown to what extent.

Since positive or negative responses are unreliable in 17% of the cases, it seems that the

ovarian hyperemia test in immature rats can be used only as a first approach to the exact diagnosis of pregnancy in equids.

Summary. The ovarian hyperemia reaction in immature rats was studied following the subcutaneous injection of equine serum from 38 mares and 4 female donkeys, 42-138 days after natural insemination. The accuracy of this 24-hour pregnancy test was 83%, failures being observed both by negative reaction in 4 pregnant cases and positive reaction in 3 non-pregnant ones.

Acknowledgment is due to M. X. Camargo, J. Vieira and S. Fontes for kindly supplying the blood samples tested.

16090

Effect of Ozonized Oxygen on Anti-A, Anti-B, and Anti-Rh Typing Sera.*

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Reports of attempts to modify agglutinating and "blocking" antibodies against human erythrocytes have recently appeared in the literature. Boyd,¹ following the lead of Tyler,² reported on the effect of photo-oxidation on isohemagglutinins, anti-A and anti-Rh in the presence of Eosin Y as a sensitizer. Photo-oxidation produced by photoflood lights gradually destroyed the agglutinating activity but did not convert any of the agglutinins studied into "inhibiting," "incomplete," or "blocking" antibodies. An exposure of 15½ hours to these photoflood lights was sufficient to destroy completely the anti-Rh agglutinin. Anti-A agglutinin, however, required an exposure of 65 to 71 hours to destroy all agglutinating activity. Although it seems likely that the action of the photoflood lights in Boyd's experiments was that of oxidation, the possibility remains, nevertheless, that some other

photo-dynamic effect may have played a part in modifying the agglutinins. It seemed desirable to us, therefore, to determine the effects on such agglutinins using a different oxidizing agent.

Materials and Methods. In this study ozone was employed to determine the effect of oxidation on isohemagglutinating antibodies, since this agent has useful attributes not possessed by other oxidizing agents. There are, for example, no added end products of the reactions other than those formed from the use of O₃. Ozone is a powerful oxidizing agent that will combine with unsaturated carbon atoms and form ozonides. The normal mode of action of ozone is solely an addition with no secondary oxidation. Furthermore, the hydrogen atoms attached to unsaturated centers are left undisturbed.

Two cc quantities of the selected sera were subjected to ozonized oxygen having a concentration of approximately one part ozone to 1000 parts of oxygen. Volume concentrations were ascertained by the iodine liberation technique in which free I is liberated from

* This work was aided by grants from Miles Laboratories, Inc., Elkhart, Ind., and the University of Utah Research Fund.

¹ Boyd, William C., *J. Exp. Med.*, 1946, **83**, 221.

² Tyler, A., *J. Immunol.*, 1945, **51**, 157.

TABLE I
Effect of Ozone on Anti-A Isoagglutinin Titer.

Anti-A isoagglutinating serum	Serum dilutions							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Untreated control	+++	+++	+++	+++	+++	+++	+++	+++
T-4 min.	+++	+++	+	+	—	—	—	—
T-8 "	—	—	—	—	—	—	—	—

+++ , ++ , + , ± = Varying degrees of agglutination of Group A erythrocytes.

— = No agglutination.

T = treated with ozonized oxygen.

TABLE II
Effect of Ozone on Anti-B Isoagglutinin Titer.

Anti-B isoagglutinin serum	Serum dilutions							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Untreated control	+++	+++	+++	+++	+++	+++	+++	+++
T-4 min.	+++	+++	+++	+++	+	±	—	—
T-8 "	+++	+++	+++	+++	+	—	—	—
T-10 "	+++	+++	+++	+++	+	—	—	—
T-14 "	—	—	—	—	—	—	—	—

+++ , ++ , + , ± = Varying degrees of agglutination of Group B erythrocytes.

— = No agglutination.

TABLE III
Effect of Ozone on Anti-A Titer of Ascaris Immune Serum.

Anti-A ascaris immune serum	Serum dilutions							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Untreated control	+++	+++	+++	+++	+++	+++	+	+
T-10 min.	+++	+++	+++	+++	+++	+	—	—
T-15 "	—	—	—	—	—	—	—	—

+++ , ++ , + , ± = Varying degrees of agglutination of Group A erythrocytes (human).

— = No agglutination.

KI by O_3 in an acidic solution. Iodine in turn was measured by titration with standard thio-sulphate using starch as an indicator. Gas flow was regulated to one liter per 3 minutes of time. Calibration of flow before and after treatment of the specimen was done using a reliable gas meter.

The serum was placed in a 75 cc beaker in order to have ample space for the resulting foam, and the gas delivered by means of a drawn glass tip. The foam was agitated to make available new foam to the active agent.

The manufacture of the ozone from O_2 was accomplished with an electrical ozonizer developed by one of us (Fenning).³ The output of a modulated 125 megacycle oscillator activated with D.C. pulses with a repetition frequency of 125/sec having a duration of 10 microseconds and a peak power of 2000 Watts was used to activate the ozonizer.

The anti-A and anti-B typing sera used in this work were obtained from group B and A subjects respectively who had been stimulated with group A and B substance by the method of Witebsky *et al.*⁴ These sera were of high titer and avidity. Anti-A immune serum prepared by inoculating rabbits with saline extracts from the cuticle of *Ascaris lumbricoides* was also used in these tests. This anti-A immune rabbit serum was of high titer and reacted specifically against human group A erythrocytes but exhibited no titer against group B or O erythrocytes.

The Rh antiserum used was the anti-D (Rh_o) 85% specificity and was absorbed and processed in the Blood Grouping Laboratory in the Department of Bacteriology at the University of Utah. It had a titer of 1:128.

All erythrocyte suspensions used were of 2% strength in terms of blood sediment. They were washed once in physiological saline and resuspended. Physiological saline was used as the diluent of both sera and erythrocytes in all tests except those in which "blocking" serum was used. In these blocking tests bovine albumin, produced by Armour and Company,

was used as the diluent for both erythrocytes and serum.

Results and Discussion. Results of these tests are shown in the tables. From the data shown in Table I it is apparent that the group A isoantibody was partially destroyed in 4 minutes treatment with ozone and completely destroyed by 8 minutes of exposure.

A group A anti-B serum of identical titer and avidity similar to the anti-A serum was treated with ozone, and the results of this experiment are shown in Table II.

From the data presented in Table II it is apparent that the group B isoantibody was completely destroyed in 14 minutes and partially destroyed in lesser periods of time.

Anti-A immune serum produced in rabbits by inoculating them with cuticle from *Ascaris lumbricoides* was treated in a similar manner as were anti-A and anti-B isoagglutinin sera. The results of this experiment are shown in Table III.

The evidence in Table III indicates that the anti-A titer of *Ascaris* immune serum was lost following 15 minutes of exposure and partially lost during lesser periods of exposure.

The effect of ozone on anti-D (Rh_o) serum is shown in Table IV.

With reference to Table IV, it is apparent that progressive partial loss of titer was present in all sera tested. Furthermore, our observations show a complete loss of agglutinating and blocking antibody titer with suitably prolonged exposures in all sera of all types so far tested.

It may also be observed that whereas the "bivalent," "heat labile," "agglutinating," "complete," D (Rh_o) antiserum was destroyed by 4 minutes treatment it took 20 minutes of the same treatment to inactivate the "univalent," "heat stable," "blocking," "incomplete," D (Rh_o) antibody. These results are in agreement with the observations of other authors^{5,6} with respect to the action of physical factors such as temperature and pressure on these 2 kinds of antisera.

³ Demonstrated A.A.A.S. exhibit, Dec., 1946, Boston, Mass.

⁴ Witebsky, E., Klendshoj, N. C., and McNeil, C., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 167.

⁵ Diamond, Louis K., *J. Lab. and Clin. Med.*, 1945, **24**, 122.

⁶ Boyd, William C., *J. Exp. Med.*, 1946, **83**, 401.

TABLE IV.
Effect of Ozone on the Titer of Anti-D (Rh₀) Typing Serum.

Rh ₀ Agglutinating & blocking anti- body serum	2% Erythrocyte suspensions in saline	Serum dilutions in saline									
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Untreated control Rh ₀ Agglutinating serum	O Rh'	—	—	—	—	—	—	—	—	—	—
	O Rh''	—	—	—	—	—	—	—	—	—	—
	O Rh ₀	+++	+++	+++	+++	+++	+++	+++	++	—	—
T-4 min. Rh ₀ agglutinating serum	O Rh'	—	—	—	—	—	—	—	—	—	—
	O Rh''	—	—	—	—	—	—	—	—	—	—
	O Rh ₀	—	—	—	—	—	—	—	—	—	—
2% Erythrocyte susp. in albumin											
Serum dilutions in bovine albumin											
Control Rh ₀ "Blocking" serum	O Rh'	++	+	+	+	—	—	—	—	—	—
	O Rh ₂	+++	+++	+++	+++	+++	+	+	+	—	—
	O Rh ₀	+++	+++	+++	+++	+++	+	+	+	—	—
T-10 min. Rh ₀ "Blocking" serum	O Rh'	—	—	—	—	—	—	—	—	—	—
	O Rh ₂	+++	+++	+++	+++	+++	+	+	—	—	—
	O Rh ₀	+++	+++	+++	+++	+++	+	+	—	—	—
T-20 min. Rh ₀ "Blocking" serum	O Rh'	—	—	—	—	—	—	—	—	—	—
	O Rh ₂	±	±	—	—	—	—	—	—	—	—
	O Rh ₀	±	—	—	—	—	—	—	—	—	—

+++ + + +, ± ± = Varying degrees of Group O Rh cells.
— = No agglutination.

TABLE V.
Effect of Ozone in Converting Anti-Rh₀ Agglutinating Antibody to Blocking Antibody.

Treatment of anti-Rh ₀ agglutinating serum	Test erythrocytes 2% suspension in saline	Serum dilutions in saline								
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Untreated	O Rh'	—	—	—	—	—	—	—	—	—
	O Rh ₂	+++	+++	+	+	+	—	—	—	—
	O Rh ₀	+++	+++	+++	+++	++	+	+	—	—
	O Rh-	—	—	—	—	—	—	—	—	—
Treated 13 min. with ozone	O Rh'	—	—	—	—	—	—	—	—	—
	O Rh ₂	—	—	—	—	—	—	—	—	—
	O Rh ₀	—	—	—	—	—	—	—	—	—
	O Rh-	—	—	—	—	—	—	—	—	—
1 drop treated serum plus 1 drop untreated	O Rh'	—	—	—	—	—	—	—	—	—
	O Rh ₂	+++	+++	+++	+++	++	+	+	—	—
	O Rh ₀	+++	+++	+++	+++	++	+	+	—	—
	O Rh-	—	—	—	—	—	—	—	—	—

+++ + + +, +, ± = Varying degrees of agglutination of Group O Rh cells.

— = No agglutination.

TABLE VI.
Comparative Effects of Air, Oxygen, and Ozonized Oxygen on Anti-Rh" Agglutinating Serum.

Treatment of anti-Rh" agglutinating serum	Test erythrocytes 2% in saline	Serum dilutions in saline					
		Undil.	1:2	1:4	1:8	1:16	1:32
Untreated control	Rh'	—	—	—	—	—	—
	Rh"	+++	+++	++	+	—	—
10 minutes exposure to air	Rh ₀	—	—	—	—	—	—
	Rh'	—	—	—	—	—	—
	Rh"	+++	+++	++	+	—	—
10 minutes exposure to oxygen	Rh ₀	—	—	—	—	—	—
	Rh'	—	—	—	—	—	—
	Rh"	+++	+++	++	+	—	—
10 minutes exposure to ozonized oxygen	Rh ₀	—	—	—	—	—	—
	Rh'	—	—	—	—	—	—
	Rh"	+++	+++	++	+	—	—
	Rh ₀	—	—	—	—	—	—

+++ + + +, +, ± = Varying degrees of agglutination of Rh" cells.

— = No agglutination.

The question still remained as to whether treatment with ozonized oxygen would convert the "bivalent" anti-Rh "agglutinating" antibody to "univalent" "blocking" antibody. Boyd¹ failed to produce such a change by exposing agglutinating sera to photoflood lights. To study the effect that ozonized oxygen would exert in this direction, anti-Rh₀ agglutinating serum, having an end titer of 1:64 against a 2% saline suspension of group O Rh₀ erythrocytes, was treated and titrated in the same manner as were the serum specimens in our previous tests. The agglutinating antibody in this serum was completely destroyed by a 13-minute treatment with ozonized oxygen. After titration had shown that the Rh₀ antibody had been destroyed, there was added to each dilution of serum and erythrocyte suspension one drop of similarly diluted untreated anti-Rh₀ serum; the tube containing the 1:2 dilution of treated serum received an additional drop of 1:2 untreated serum, the 1:4 dilution received a drop of 1:4 serum and so on. Each tube now contained one drop of treated and diluted serum, one drop of 2% group O Rh₀ erythrocyte suspension, and one drop of untreated serum of the same dilution as the treated serum. The contents of the tubes were mixed by shaking the rack containing the tubes and all were then again incubated in the 37°C water bath. The results of this test are shown in Table V.

It is clear from Table V that, although the agglutinating antibody was destroyed by treatment with ozone, there was nothing produced in it which inhibited the action of the untreated anti-Rh₀ agglutinating serum when, subsequently, one drop of each dilution of the untreated serum was added to each tube and then incubated in the water bath at 37°C for an additional hour. There appears to be no conversion of anti-Rh₀ agglutinating

antibody to anti-Rh₀ blocking antibody by treatment with ozone.

In order to evaluate the potential destructive effects of foaming on proteins and related isohemagglutinins, anti-Rh serum was subjected to equivalent 10-minute flows of air, O₂, and ozonized O₂. The control agglutinating titer was 1:8. Following the bubbling of air the agglutinating titer was 1:4; for oxygen, 1:4; whereas the agglutinating titer for comparable exposure to ozonized oxygen was 1+ for the undiluted treated specimen. It will be noted in Table VI that some change was evident in the terminal titer. The intensity of agglutination for the lower dilutions showed no change with air and oxygen whereas a profound change was present with ozonized oxygen. Oxidation as effected by ozone in combination with oxygen is more potent than oxygen alone.

With the use of ozonized oxygen considerable macroscopic evidence of denaturation was evident. In most instances with prolonged exposure there developed a gummy, gelatinous precipitate which adhered to the glass rod and sides of the container. With short exposures macroscopic evidence of this precipitate was frequently absent.

Summary: It has been demonstrated that the blood group isoagglutinins, immune anti-A agglutinin, and Rh agglutinating and blocking antibodies are inactivated or destroyed relatively rapidly with 1:1000 dilution of ozone in oxygen.

So far as Rh agglutinating and blocking antibodies are concerned, our results are in essential agreement with the observations of others with respect to the action of physical factors such as temperature and pressure.

There appears to be no conversion of Rh₀ agglutinating antibody to Rh₀ blocking antibody by treatment with ozone.

Effect of Cytochrome C Therapy on Altitude Tolerance of Normal Rats.

WILLIAM R. CHRISTENSEN AND MARSHALL CLINTON, JR.

(Introduced by A. Baird Hastings.)

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Previous studies have indicated that the ability of normal rats to withstand severe anoxia can be improved by a variety of procedures. Animals rendered polycythemic by the administration of cobalt were capable of increased work performance under conditions of severe anoxia.¹ Administration of a concentrated extract of adrenal cortex in sesame oil was followed by a marked improvement in ability to withstand short exposures to ordinarily lethal altitudes.² Animals given a 2-hour conditioning exposure to pressures equivalent to 18,000 or 26,000 feet altitude daily for 7 or 30 days developed improvement in their ability to withstand exposure to ordinarily lethal altitudes.³ In these later studies, the improvement was most marked after conditioning at the higher altitude, and was accompanied by both polycythemia and adrenal gland hypertrophy. However, the improvement could not be explained solely on the basis of either polycythemia or adrenal gland hypertrophy, since it occurred following conditioning at lower altitudes, which failed to influence organ weight or morphology, and there was a lack of correlation between hematocrit increase and the survival rate of the conditioned animals. It is apparent, therefore, that the mechanisms responsible for the increased altitude tolerance found in the above-noted experiments are not entirely clear. In view of this circumstance, it has been considered advisable to appraise the effect of parenterally administered cytochrome

C upon altitude tolerance. This was undertaken because of the recently demonstrated influence of adrenalectomy on cytochrome oxidase and cytochrome C concentration⁴ and because of the beneficial influence of parenterally administered cytochrome C on patients suffering from inadequate oxygenation of their tissues.^{5,6,7}

Methods. Young, male, white rats of approximately 200 g average weight were used in these studies. The animals were maintained in wire mesh cages on a standard diet of Purina dog chow. Access to food and water was unrestricted until 5 hours prior to exposure to anoxia; after this, water only was allowed. Five mg of cytochrome C in 2 cc of isotonic saline were administered to the experimental animals in a single intraperitoneal injection 45 minutes prior to altitude exposure.* The control animals were handled in the same manner as the treated group, except that an equal volume of physiological saline was substituted for the cytochrome C solution.

The cytochrome C was isolated according to the technique of Keilin and Hartree which yields a product containing .34% Fe.⁸ The concentration of cytochrome in the injected material was determined by spectrophotometric analysis. The amount given is com-

¹ Dorrance, S. S., Thorn, G. W., Clinton, M., Jr., Edmonds, H. W., and Farber, S., *Am. J. Physiol.*, 1943, **139**, 399.

² Thorn, G. W., Clinton, M., Jr., Davis, B. M., and Lewis, R. A., *Endocrinology*, 1945, **36**, 381.

³ Thorn, G. W., Clinton, M., Jr., Farber, S., and Edmonds, H. W., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 59.

⁴ Tipton, S. R., *Endocrinology*, 1944, **34**, 181.

⁵ Proger, S., *Bull. N. E. Med. Center*, 1945, **7**, 1.

⁶ Proger, S., and Dekaneas, D., *Bull. N. E. Med. Center*, 1945, **7**, 149.

⁷ Proger, S., and Dekaneas, D., *Science*, 1946, **104**, 389.

* It has been reported by Proger and Dekaneas⁷ that intravenous, intramuscular, and intraperitoneal injections of cytochrome C increase the blood and organ content of cytochrome C.

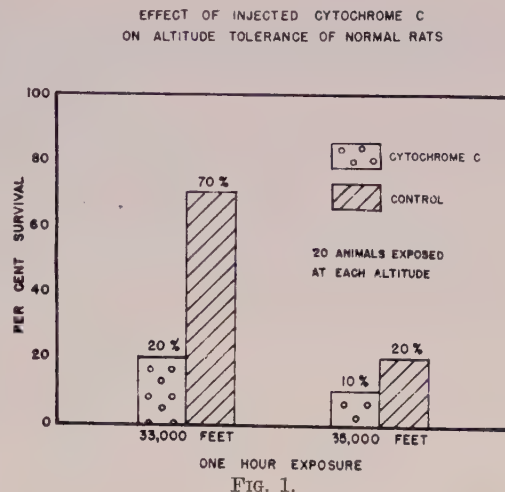
⁸ Keilin, D., and Hartree, E. F., *Biochem. J.*, 1945, **39**, 289.

parable to that used by Proger and Dekaneas in their studies.^{5,6}

The acute altitude survival test has been described in detail elsewhere.^{2,3} Exposure was carried out in a decompression chamber with sufficient air flow to prevent any perceptible increase in carbon dioxide content. The survival rates reported represent the percentage of animals alive at the conclusion of the test, since no delayed deaths were noted. All animals were exposed for one hour at the test altitude. Moreover, they were exposed simultaneously, thus eliminating any possible differences in survival rate due to variations in temperature and humidity within the chamber or such other unpredictable variables as handling and feeding.

Results and Discussion. The results of exposure with and without parenteral cytochrome C were as shown in Table I. A graphic presentation is given in the accompanying chart.

It is apparent that cytochrome C was not effective in protecting the intact rat against severe anoxia. If injected cytochrome C is capable of increasing the ability of tissues to utilize oxygen under conditions of anoxia, one might expect administration of this respiratory enzyme to increase the ability of the intact organism to withstand the anoxia of high altitude exposure. The present experiments give no indication as to why this expected action did not occur. It is quite possible that the cytochrome C, being a pro-



tein whose molecular weight is at least 13,000, was unable to penetrate intact cells. However, there are indications that even had this occurred, it would not have affected the oxygen consumption of the cells.⁹

Summary. This report presents data concerning the ability of rats treated with cytochrome C to withstand the anoxia of high altitude exposure. The survival rates of experimental animals given an intraperitoneal injection of 5 mg of cytochrome C in isotonic saline were compared with those of control animals treated with a similar amount of saline. Groups of control and experimental animals were exposed simultaneously for periods of one hour. Altitudes of 33,000 and 35,000 feet were employed. In both instances, the survival rate was higher in the control group. It is concluded, therefore, that the intraperitoneal injection of cytochrome C is not effective in protecting rats against the anoxia of high altitude exposure.

TABLE I.
10 animals in each experiment.

Exposure altitude	Treatment	% survival
ft		
33,000	Normal saline—2 cc	70
"	Cytochrome C—5 mg	20
35,000	Normal saline—2 cc	20
"	Cytochrome C—5 mg	10

⁹ Shack, J., *J. Nat. Cancer Inst.*, 1943, **3**, 389.

Chemotherapy of Experimental Tuberculosis with Benzothiazole Derivatives.*

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In the following experiments a new class of tuberculostatic agents is described. A systematic evaluation of the bacteriostatic action of various benzothiazole derivatives on the growth of tubercle bacilli *in vitro* was attempted. Many of the compounds were also investigated *in vivo* for their therapeutic action on tuberculous animals. A few benzothiazole derivatives have previously been evaluated by Everitt and Sullivan¹ for their fungicidal action and as antiviral agents by Kramer and associates.² The authors³ have reported the effect of several benzothiazole sulfones in experimental tuberculosis.

Methods. The bacteriostatic studies were initially performed on the avirulent strain of human tubercle bacilli, No. 607 of the American Type Culture Collection, using Lowenstein's liquid asparagin media, at a pH of 7.5. Many of the compounds were also tested on the virulent human strain, H37Rv, in Proskauer and Beck media. The chemicals were dissolved in propylene glycol to make 0.5% solutions. Subsequent dilutions were made directly into the aqueous media, without further heating. Each tube was inoculated with one loopful of fresh culture of tubercle bacilli. The avirulent cultures were read at the end of 3 days; the virulent cultures at 18 days. The highest bacteriostatic dilution was

taken as that dilution in which there was less than 50% of control growth.

Animal Experiments. Nine to 12 guinea pigs, averaging 400 g in weight, were used to evaluate each compound. The animals were inoculated in the left groin with 0.03 mg of H37Rv tubercle bacilli. The drugs were administered orally, suspended in water, by dropper once daily. Therapy was started on the day of infection. The dosage of the drugs was increased gradually for the first 2 weeks of the experiment. The dosage of all drugs approached the chronic maximum tolerated dose. The experiments were terminated at the end of 6 weeks. Pathology was evaluated numerically, based on a maximum of 4 for each organ (glands, spleen, liver and lungs); with a maximum of 16 for all organs. Hemoglobin and weight determinations were made at the end of the experiments.

Results. The *in vitro* bacteriostatic results are recorded in Table I. The benzothiazole ethers (Nos. 12 to 19 incl.) were the most extensively investigated group. The compound 6-amino-2-(n)butoxybenzothiazole (No. 16) was bacteriostatic in extremely high dilution (1:40,000,000) on H37Rv. This activity was a thousand times as great as its bacteriostatic effect on the avirulent strain 607. All compounds tested were more bacteriostatic against the virulent culture. Five other types of benzothiazole derivatives were tuberculostatic in low concentrations: 6,2-diaminobenzothiazole (No. 20); 6-amino-2-chlorobenzothiazole (No. 24); 6-aminobenzothiazole (No. 3); 6-amino-2-mercaptobenzothiazole (No. 6) and 6-amino-2-methylbenzothiazole (No. 8). In general, the compounds were bacteriostatic rather than bactericidal. Compounds Nos. 16, 20 and 24 were tested in the presence of 20% defibrinated horse blood. They retained most of

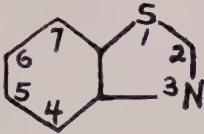
*The present report is part of a cooperative investigation on tuberculosis, and has been supported by funds provided by the Committee on Medical Research of the National Tuberculosis Association, the California Tuberculosis Association, the San Francisco Tuberculosis Association, and the Columbia Foundation of San Francisco.

¹ Everitt, E. L., and Sullivan, M. X., *J. Wash. Acad. of Sci.*, 1940, **30**, 125.

² Kramer, S. D., Greer, H. A., and Szobel, D. A., *J. Immunol.*, 1944, **49**, 273.

³ Freedlander, B. L., and French, F. A., *Am. Rev. Tuberc.*, in press.

TABLE I.
 Tuberculostatic Action on Strains 607 and H37Rv.

No.	Compound	Formula	Mg %	
			607	H37Rv
1	Benzothiazole		10.	5.
2	2-Aminobenzothiazole	$C_7H_4NSNH_2$	>10.	
3	6-Nitrobenzothiazole	$C_7H_4NSNH_2$	0.25	0.1
4	6-Nitrobenzothiazole	$C_7H_4NSNO_2$	10.	
5	2-Mercaptobenzothiazole	C_7H_4NSSH	5.	
6	6-Amino,2-mercaptobenzothiazole	$H_2NC_7H_3NSSH$	5.	0.1
7	2-Methylbenzothiazole	$C_7H_4NSCH_3$	10.	
8	6-Amino,2-methylbenzothiazole	$H_2NC_7H_3NSCH_3$	0.1	0.01
9	6-Nitro,2- "	$O_2NC_7H_3NSCH_3$	10.	
10	2-Phenylbenzothiazole	$C_7H_4NSC_6H_5$	10.	
11	2-Chlorobenzothiazole	C_7H_4NSCl	5.	
12	6-Amino,2-ethoxybenzothiazole	$H_2NC_7H_3NSOC_2H_5$	10.	0.7
13	6-Nitro,2- "	$O_2NC_7H_3NSOC_2H_5$	>10.	
14	6-Amino,2-isopropoxybenzothiazole	$H_2NC_7H_3NSOCH(CH_3)_2$	2.5	0.35
15	6-Nitro,2- "	$O_2NC_7H_3NSOCH(CH_3)_2$	10.	
16	6-Amino,2-(n)butoxybenzothiazole	$H_2NC_7H_3NSOC_4H_9$	2.5	0.0025
17	6-Nitro,2- "	$O_2NC_7H_3NSOC_4H_9$	1.	
18	6-Amino,2-butoxyethoxybenzothiazole	$H_2NC_7H_3NSOC_2H_4OC_4H_9$	>10.	
19	6-Nitro,2- "	$O_2NC_7H_3NSOC_2H_4OC_4H_9$	5.	
20	6,2-Diaminobenzothiazole	$H_2NC_7H_3NSNH_2$	0.25	0.01
21	6-Nitro,2-aminobenzothiazole	$O_2NC_7H_3NSNH_2$	2.5	
22	6-Amino,2-hydroxybenzothiazole	$H_2NC_7H_3NSOH$	10.	
23	6-Nitro,2- "	$O_2NC_7H_3NSOH$	2.5	
24	6-Amino,2-chlorobenzothiazole	$H_2NC_7H_3NSCl$	0.025	0.0025
25	6-Nitro,2- "	$O_2NC_7H_3NSCl$	1.	
26	6'-Aminobenzothiazolyl,2'-(N)-morpholine	$H_2NC_7H_3NSNC_4H_8O$	>10.	
27	6'-Nitrobenzothiazolyl,2'-(N)-morpholine	$O_2NC_7H_3NSNC_4H_8O$	>10.	
30	5-Amino-2-isopropoxypyridine	$H_2NC_5H_4NOCH(CH_3)_2$	1.	0.1

The above compounds were prepared in this laboratory under the direction of Dr. F. A. French with the following exceptions: Nos. 1, 2, 5, 6, 7, 10 and 11 were obtained from the Eastman Kodak Co. (highest purity).

Control experiments were performed with propylene glycol alone in 1:50 dilution. No bacteriostatic effect was noted.

their bacteriostatic activity. Compounds Nos. 16 and 20 were tested against the following organisms: *S. aureus*, *E. coli*, *Proteus vulgaris*, *Streptococcus pyogenes* and *Ps. pyocyaneus*. The results were essentially negative.

The *in vivo* results are summarized in Table II. 6-amino-2-(n)butoxybenzothiazole and 6,2-diaminobenzothiazole were highly effective. These compounds were approximately twice as active as 4,4'-diaminodiphenylsulfone (No. 28) and about $\frac{3}{4}$ as effective as streptomycin (No. 29). 6-Amino-2-isopropoxy and 6-amino-2-ethoxybenzothiazole (Nos. 14 and 12) were also significantly active therapeutically. Animals treated with

6,2-diaminobenzothiazole showed no enlargement of the thyroid gland. This is of interest because this compound contains a thio-ureoid structure similar to that found in 2-aminothiazole which has been reported to inhibit thyroid activity.⁴

Discussion. 6-Aminobenzothiazoles with 5 types of substituents in the 2- position have been found to be highly active against tubercle bacilli. The 2- substituents included alkoxy, alkyl, amino, chloro and mercapto. The invariant characteristic of the group is the possession of an aromatic amino group

⁴ Williams, R. H., Kay, G. A., and Solomon, B., *Am. J. Med. Sci.*, 1947, **213**, 198.

TABLE II.
 Summary of *in vivo* Experimental Data.

No.		Avg daily dosage mg per animal	*Path. ratio, Controls	Relative wt gain, g deviation from controls	Hglb. % deviation from controls
			Treated		
12	6-Amino-2-ethoxybenzothiazole	50-150	2.40	-87.	+14.
13	6-Nitro-2- "	50-150	1.60	-72.	0.
14	6-Amino-2-isopropoxybenzothiazole	50-150	2.28	-58.	+14.
16	6-Amino-2-(n)butoxybenzothiazole	50-100	3.05	-67.	-3.
18	6-Amino-2-butoxyethoxybenzothiazole	75-125	1.75	-80.	0.
19	6-Nitro-2- "	50-130	1.04	+25.	0.
20	6,2-Diaminobenzothiazole	50-150	3.03	-68.	0.
24	6-Amino-2-chlorobenzothiazole	50-100	1.59	-50.	8.
28	4,4'-Diaminodiphenylsulfone†	50-100	1.66	-32.	-20.
29	Streptomycin†	20 (hypo)	3.9	-5.	-5.
30	5-Amino-2-isopropoxypyridine	30	1.14	-137	-9.

* "Pathological Ratio" is the result of the average numerical pathological rating of the control animals divided by that of the treated animals.

† Diaminodiphenylsulfone and streptomycin are included for comparison.

in the 6- position and of an activating substituent in the 2- position. Kumler and Daniels^{5,6} proposed a theory of activity of sulfonamides and aminoacridines in which the reactive character of the aromatic amine group is correlated with bacteriostatic activity. The aromatic amino group must be present, of course, in a molecule of suitable overall structure. This theory may be applied to these new tuberculostatic agents. The 6-amino group is an aromatic amino group and the 2- substituents may be regarded as activating groups. Using 6-amino-benzothiazole, where the 2- position is occupied by hydrogen, as a base level, one may calculate activity ratios with strain H37Rv as the test organism. With the following 2-substituents the activity ratios are: CH₃ -10, SH -1, OC₂H₅ -0.14, NH₂ -10, OC₄H₉(n) -40. In a previous paper³ it was shown that a 6-aminobenzothiazole substituted with 2-arylsulfonyl groups, which are electroacceptors, had only a low order of activity. The present compounds with electrodonor groups in the 2- position are as much as 1,000 times as active. Unfortunately, as yet, no adequate quantitative measure of the reactive character of the aromatic amine group is available. Qualitative comparisons can be made

if adequate allowance is made for other important factors affecting drug activity.

In the case of the 6-amino-2-alkoxybenzothiazoles it is apparent that the distribution coefficient is of considerable significance and this effect is much more noticeable with H37Rv than with strain No. 607. 6-Amino-2-ethoxy, isopropoxy, and (n)butoxybenzothiazoles all have about the same activity against strain No. 607 but the butyl ether is 300 times as active as the ethyl ether against strain H37Rv. The generic similarity of these benzothiazole ethers to the pyridine and other ether-amines studied by Feinstein, Friedman and co-workers⁷⁻¹¹ suggests that certain generalizations of some validity concerning both groups of compounds can be made. They found an optimum activity with ether chains of 4 to 6 carbon atoms. In addition it is noticeable from their data that the

⁷ Friedman, H. L., Braitberg, L. D., Tolstouhov, A. V., and Tisza, E. T., *J. Am. Chem. Soc.*, 1947, **69**, 1795.

⁸ Braitberg, L. D., Robin, M., Friedman, H. L., and Tisza, E. T., *J. Am. Chem. Soc.*, 1947, **69**, 2005.

⁹ Friedman, H. L., Braitberg, L. D., Tolstouhov, A. V., and Tisza, E. T., *J. Am. Chem. Soc.*, 1947, **69**, 1204.

¹⁰ Feinstein, W. H., Friedman, H. L., Rothlauf, M. V., Kelly, A. M., and Williams, R. D., *J. Pharm. and Exp. Therap.*, 1947, **89**, 153.

¹¹ Feinstein, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 153.

⁵ Kumler, W. D., and Daniels, T. C., *J. Am. Chem. Soc.*, 1943, **65**, 2190.

⁶ Daniels, T. C., personal communications.

symmetry of the ether group is very important. The chain length and symmetry of the ether group may very well maximize a tendency for proper molecular orientation and binding at some very important intracellular interface or enzyme locus. As evidence of the importance of symmetry the data of Friedman *et al.*⁹ may be cited. With the 2-alkoxy-5-aminopyridines, using the n-amyl ether as unity the activities against strain No. 607 are: i-amyl -0.03, 3-n-amyl -0.5, n-butyl -1, i-butyl -.07, sec-butyl -0.016. These orientation factors may be much more important than the commonly known one of an optimal distribution coefficient, dependent on chain length, producing a maximal drug concentration in some lipid phase.

The high order of specificity of some of the benzothiazole derivatives for strain H37Rv relative to strain No. 607 is encouraging. In the case of 6-amino-2-(n)-butoxybenzothiazole the activity ratio for the two strains is 1000. With other benzothiazole derivatives the ratio varies from 2 to 50. With the aryl-sulfones (extensively studied) the activity ratios were close to unity.

Those benzothiazole derivatives which were tested *in vivo* showed a low or moderate chronic toxicity. In the case of 6-amino-2-(n)-butoxybenzothiazole a definite hypnotic effect appeared at the maximum tolerated dose. This is not unexpected for an ether-amine nor for a compound which may be regarded as a cyclic thiocarbamic ester. Hemotoxic effects which are so prominent with derivatives of diaminodiphenylsulfone were absent with the benzothiazole compounds. For compar-

ison of toxicity and activity, 2-isopropoxy-5-aminopyridine (No. 30) (somewhat comparable to 6-amino-2-isopropoxybenzothiazole of this series) was evaluated *in vivo*. While this compound is not at the pyridine series optimum the comparison to equivalent benzothiazole compounds is significant. It may be noted that much smaller dosages of the pyridine ether were tolerated, marked liver damage occurred, and no significant effect on the infection was noticed.

Many types of compounds possessing active aromatic amino groups are tuberculostatic. Feinstone, Friedman and co-workers found many amino pyridines, pyrimidines and benzenes to be active. The authors¹² have noted fair activities with some amino acridines and phenothiazines. It may be suspected that some tuberculostatic activity is possessed by a great variety of amino-cyclic compounds in which the amino group is aromatic in character and is suitably activated.

Further studies of benzothiazole derivatives, including solublized derivatives of 6-amino-2-(n)-butoxybenzothiazole, are being undertaken.

Summary. 1. A new class of highly tuberculostatic agents of low toxicity is described. This group comprises many 2,6- substituted derivatives of benzothiazole. 2. Several of these derivatives are active therapeutic agents in guinea pig tuberculosis. 3. Structure activity relations and possible modes of action are discussed.

¹² French, F. A., and Freedlander, B. L., unpublished data.

Influence of X-rays on Respiration of Nuclei of Fowl Erythrocytes.*

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In recent years a series of publications have appeared dealing with the influence of X-rays on respiration. Williams and Scheard¹ demonstrated increased oxidation in irradiated frog's skin. Lacassagne² working with yeasts also observed increased O₂ consumption after irradiation.

In a previous paper³ we have demonstrated that fowl-erythrocytes irradiated with X-rays consume more oxygen than do unirradiated red cells. The purpose of the present study was to investigate the respiration of the irradiated nuclei of fowl erythrocytes and to compare the results with those obtained with whole erythrocytes.

Methods. (a) Preparation of nuclei. Nuclei were prepared according to the technique of Dounce and Lan. White Leghorn hens were bled by cardiac puncture and 20 cc of blood were sterilely withdrawn into 2 ml of 3% solution of Na citrate. The erythrocytes were centrifuged twice washed in 0.9% saline and the sedimented cells were resuspended in saline to the original volume of the blood. In order to induce hemolysis a solution of 0.15 g of saponin in 5 ml of an 0.11 M solution of phosphate (pH 7) was added to each 100 ml of erythrocyte suspension. After hemolysis was completed (within 2-3 minutes), the hemolysate was centrifuged and subsequently washed 5 or 6 times by centrifuging in 0.9% saline and was finally resuspended in this solution up to a concentration of approximately 10-18 mill/mm³. The nuclei

were then counted in a counting chamber.

(b) Irradiation. The X-ray tube used in these experiments was operated at a tension of 35 KV at a current of 15 mA with a copper anticathode. The window was aluminum foil 30 μ in thickness. Absorption analysis showed that the rays which penetrated through the window were mainly copper X-rays. The distance between the vessel in which the nuclei were irradiated and the focus was 50 mm and the X-ray intensity was about 60,000 r/min. Irradiation was performed on 0.5 ml portions of the nuclei suspensions in flat-bottomed, shallow glass dishes of 15 mm diameter. The suspension layer did not exceed 3 mm in thickness. Irradiation doses of 0.5 Mil., 1 Mil., and of 1.5 Mil. were applied.

(c) Measurement of respiration. Respiration was measured in a Warburg apparatus at 39-40°. Two ml of suspension were used per vessel for each estimation. Five to six 0.5 ml portions had, therefore, to be irradiated consecutively and combined. Control estimations of non-irradiated nuclei from the same sample of suspended nuclei were carried out in every case. The samples to be irradiated, as well as those already irradiated and the controls were kept under identical conditions of temperature throughout. The first reading was made 20 minutes after inserting the manometers in the water-bath (about half an hour after the termination of irradiation). Readings were taken initially at intervals of 30 minutes and later at intervals of 60 minutes.

Results and Discussion. As Dounce has already shown chicken erythrocyte nuclei continue for some days to exhibit a weak but demonstrable respiration. In our study this respiration of isolated nuclei was $\frac{1}{8}$ to $\frac{1}{10}$ of that of whole erythrocytes. Absolute values varied with individual chickens. X-rays had

* Aided by a grant of the British Empire Cancer Campaign.

¹ Williams, M. M. D., and Scheard, C., *Protoplasma*, 1932, **15**, 396.

² Lacassagne, Schoen et Beraud, *Ann. des Fermentations*, 1939, **5**, 124.

³ Frankenthal, L., and Back, A., *Biochem. J.*, 1944, **38**, 351.

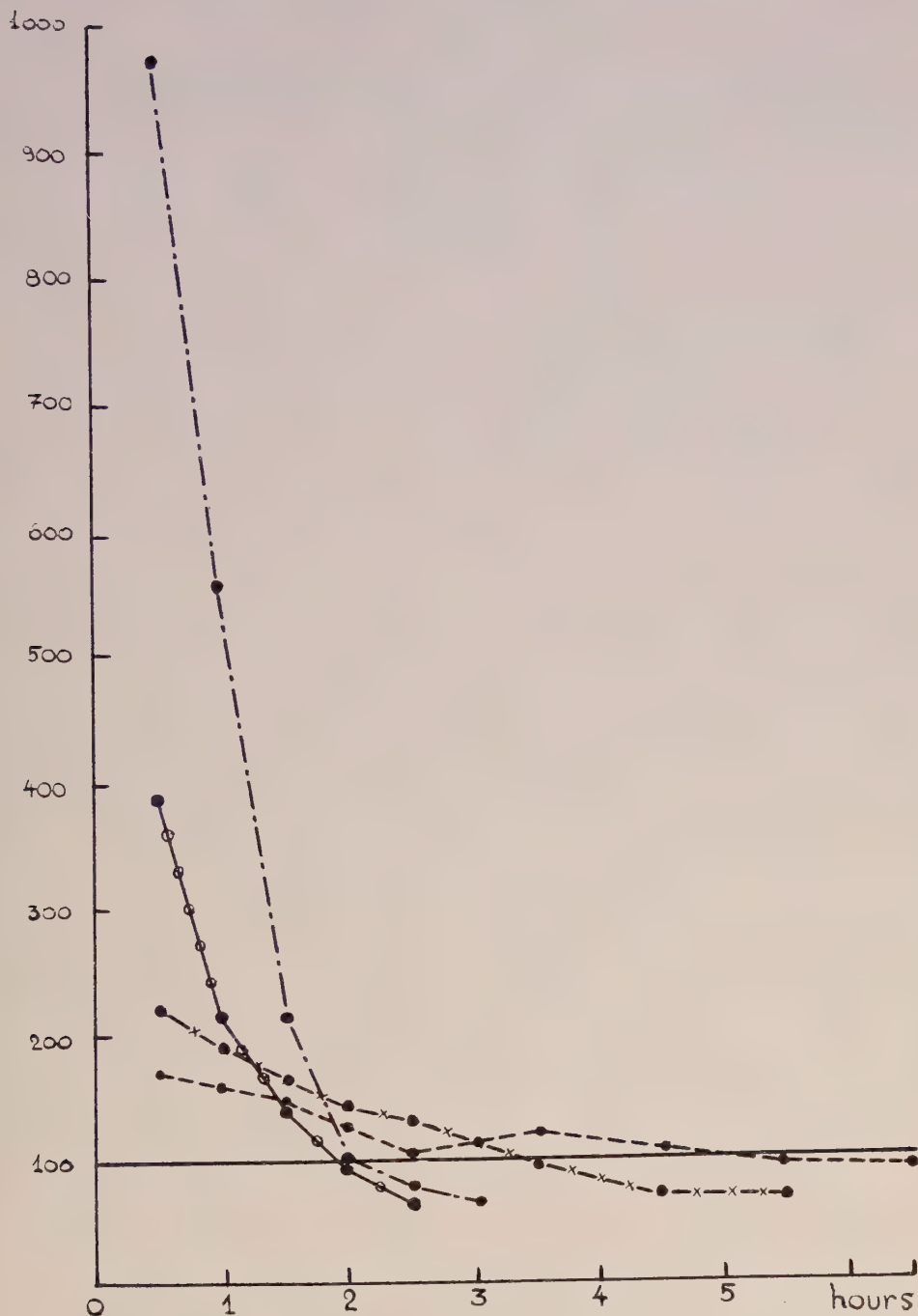


FIG. 1.

Effect of X-radiation on the respiration of nuclei of fowl erythrocytes and of whole erythrocytes. The data given are the O_2 consumption in the experimental vessels, expressed as a percentage of the O_2 consumption observed in the control vessels at a given time.

- Non-irradiated nuclei or erythrocytes.
- Nuclei irradiated with 500,000 r.
- — — — — Nuclei irradiated with 1,000,000 r.
- · · · · Erythrocytes irradiated with 500,000 r.
- × — Erythrocytes irradiated with 1,000,000 r.

TABLE I.

Influence of Irradiation on O₂ Consumption of Nuclei of Fowl Erythrocytes.
 μ l O₂ Consumed by 2 ml of Nuclei Suspension 10 Mill Nuclei/1 mm³ After 30 to 180 min.

Irradiation dose (in millions r)	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.
0.5	22.9	41.5	52.1	59.1	64.4	
0	5.9	14.4	22.0	29.6	38.1	
1	68.8	101.2	120.5	131.2	140.7	148.9
0	7.1	14.7	23.6	34.8	47.1	59.6
1.5	16.7	28.6	35.9	41.2	46.6	50.5
0	2.0	4.0	8.0	12.6	17.8	23.9

an accelerating effect on the respiration of nuclei and this effect was far more pronounced than that on whole erythrocytes as may be seen in Fig. 1. Thus, the effect of $\frac{1}{2}$ million r on isolated nuclei is about twice as great and the effect of one million r is more than 4 times as great as the corresponding effects on whole erythrocytes.

In Fig. 1 we give the rate of O₂ consumption of normal and of irradiated nuclei and of whole erythrocytes irradiated and non-irradiated. The rate of respiration of non-irradiated erythrocytes and that of non-irradiated nuclei was arbitrarily designated as 100 (the horizontal base line). Values for irradiated nuclei observed at the same times were computed relatively to this normal value. The curves thus represent percentage increase or decrease in the respiration of irradiated nuclei compared with non-irradiated controls, plotted against time. The same figure also presents the curves describing the intensified respiration of whole erythrocytes irradiated also with identical doses of $\frac{1}{2}$ and one million r. The fact that the curves for nuclei for both doses fall sharply and that in both cases the normal is reached simultaneously is striking.

Table I presents a series of typical experiments with different doses of X-rays. These figures show that after exposure to any of the given doses the intensity of respiration of the irradiated nuclei is initially high, becomes gradually weaker and falls to below normal after 3 hours. A similar, though somewhat delayed course is taken by the respiration of whole erythrocytes after irradiation, in which case the process of inhibition sets

in much later.

It is still impossible to explain the process of X-ray activation described by various authors. The observed difference between the respirations of irradiated erythrocytes and irradiated nuclei alone, however, may be due to the protective effect of cytoplasmic proteins against the influence of X-rays. In order to verify this assumption we added 0.5% albumin or hemolysate to the nuclei before irradiation, thereby considerably weakening the stimulation.

Dale,⁴ Friedewald,⁵ Evans⁶ and others have described such a protein effect on viruses, enzymes and sea-urchin sperm. It may be assumed that the proteins present in erythrocytes (globulin, hemoglobin, etc.) also exert a similar protective effect on the influence of X-rays on the respiration of whole erythrocytes.

Summary. 1. The effect of X-ray irradiation on the respiration of the nuclei of fowl erythrocytes has been investigated. 2. The respiration of these nuclei was temporarily stimulated by treatment with doses of 500,000 to 1,500,000 r. This stimulation of nuclear respiration was more pronounced than that obtained with whole erythrocytes. 3. The addition of hemolyzed blood or albumin to the suspension of nuclei before irradiation has a protective effect against the activating influence of X-rays.

⁴ Dale, W. M., Meredith, W. J., and Tweedie, M. C. K., *Nature*, 1943, **151**, 280.

⁵ Friedewald, W. F., and Anderson, R. S., *J. Exp. Med.*, 1941, **74**, 463.

⁶ Evans, T. C., Slaughter, J. C., Little, E. P., and Failla, G., *Radiology*, 1942, **39**, 663.

Relationship of Weight, Venous Pressure and Radiosodium (Na^{22}) Excretion in Chronic Congestive Heart Failure.*

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During the course of tracer studies with Na^{22} † in 2 patients with chronic congestive heart failure and one normal subject, the relationship of weight, venous pressure and sodium excretion was observed.

Each subject received an intravenous injection of a tracer dose of Na^{22} (activity of 12,500,000 counts per minute¹). Over a 2-month period, all urine samples were collected separately and the concentration of radio-sodium determined for each, as well as for daily samples of blood serum. All counts were corrected so as to correspond to an initial injection of Na^{22} with an activity of 20,000,000 counts per minute per 100 lb body weight. Weights and venous pressures² were recorded daily.

Results. (1) *Behavior of weight, venous pressure and sodium excretion with the subjects on a low sodium diet.* In all 3 subjects during a 22-day period of low sodium intake (<1.7 g NaCl/day), the weight and venous pressure varied concordantly, although the daily changes were not always concordant (Fig. 1, 2, and 3). The associated changes in sodium excretion were inversely related to those of weight and venous pressure. The patient whose weight and venous pressure were decreasing excreted the largest per cent of the injected Na^{22} during this period, while the

least was excreted by the patient whose weight and venous pressure were increasing.

(2) *Effect of adding sodium chloride to the diet.* Twelve grams of NaCl per day were added to the diet of the normal subject and the patient with congestive heart failure who was recovering (Patient No. 1). This resulted in a marked increase in the sodium clearance and in the sodium excretion of the normal subject, but there was only a slight increase in the clearance without an increase in the total Na^{22} excretion in the patient with heart failure. There was a slight increase in the weight and venous pressure associated with the decrease in total Na^{22} output in the patient with congestive heart failure.

(3) *Effect of Mercuhydrin (mercurial diuretic).* The most pronounced effects of Mercuhydrin (the sodium salt of methoxyoximercuripropylsuccinylurea with theophylline)‡ were an increase in sodium clearance and a decrease in weight in all subjects. Other effects included an increased excretion of water and sodium, usually with a proportionately greater increase in sodium excretion than in water output. Often there was a decrease in venous pressure. It may be noted that frequently during the periods following the diureses due to Mercuhydrin, the excretion of sodium and water was less than had occurred during and just prior to the diureses.

(4) *Relation of sodium excretion to fluid output.* In general, the day to day fluctuations in the excretion of water and of sodium were concordant, the latter fluctuating more, particularly following Mercuhydrin.

(5) *General trends.* During the 60-day period of observation both the venous pressure and weight remained within a constant range

* Aided by a grant from the Life Insurance Medical Research Fund, a War Contract No. W-49-007-MD-389, the Helis Institute for Medical Research, and the Mrs. E. J. Caire Fund for Research in Heart Disease.

† Made available through cooperation of Professors M. T. Kamen and A. L. Hughes of the Washington University Cyclotron.

¹ Reaser, Paul B., and Burch, George E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 543.

² Burch, G., and Winsor, T., *J. A. M. A.*, 1943, **123**, 91.

‡ Supplied by Lakeside Laboratories, Milwaukee, Wis.

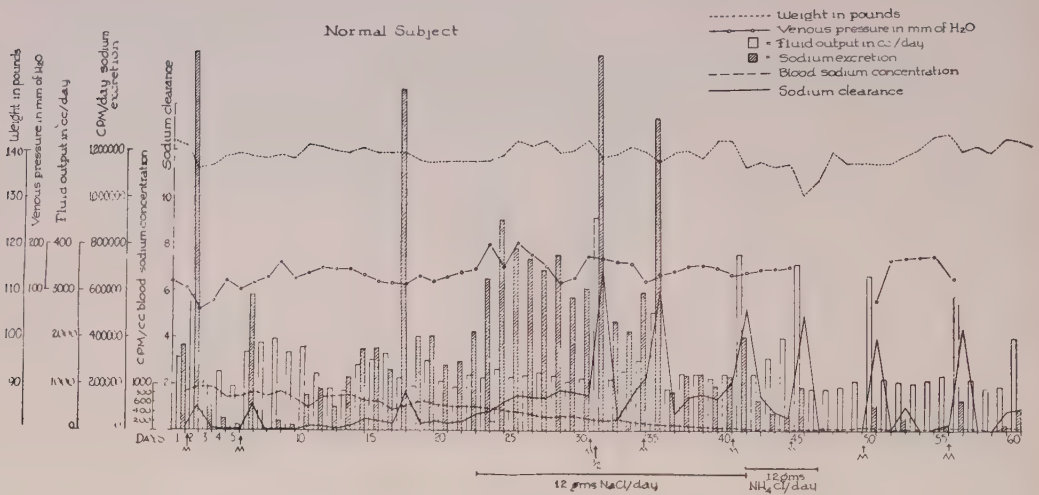


FIG. 1.

Variations in weight, venous pressure, fluid output, radiosodium (Na^{22}) excretion, blood radiosodium concentration and blood radiosodium clearance in a normal 37-year-old negro male. The weight and venous pressure showed daily variations within a narrow range. The addition of 12 g of NaCl per day to the diet resulted in an increase in blood radiosodium clearance and total radiosodium excretion. Intramuscular injections (2 cc) of Mercurhydrin (M) resulted in increases in radiosodium clearance, fluid output and radiosodium excretion. One-half the injected radioactive sodium was excreted in 30 days and 69% was excreted by the end of the study (60 days). The subject was on a low sodium diet except from the 22nd to the 41st day. On the fifteenth day K^{42} was administered for other study purposes.

for the normal subject (Fig. 1), decreased in the patient recovering from failure (Fig. 2) and increased in the patient decompensating (Fig. 3).

The normal subject excreted 50% of the injected Na^{22} in the urine in 30 days and 69% by the end of 60 days (Fig. 4). The patient rapidly improving had excreted 50% in 25 days and 64% by the end of 60 days, while the patient decompensating had excreted only 42% at the end of the 60 days.

For a period of 5 hours following one of the injections of Mercurhydrin (on the 35th day) several serial determinations of weight and venous pressure were made. The behavior of weight, venous pressure, water output, Na^{22} excretion and Na^{22} clearance were similar for the briefer period to that described for the longer intervals. That is, weight and venous pressure were concordant and were inversely related to fluid and Na^{22} excretion and clearance.

Comments. The findings in this study were in accord with the generally accepted ideas of congestive heart failure, that is, the pa-

tients with congestive heart failure were found to excrete less than normal amounts of Na and water during the period that the failure was advancing, but during the period of improvement the excretion of salt and water exceeded that observed in the normal subject. The patient recovering from congestive heart failure excreted more sodium when on a low sodium diet than when on a high sodium one, while the reverse was true for the normal subject (Fig. 1, 2). Mercurial diuretics, digitalis and ammonium chloride were among the other agents, the influences of which were observed during the 60 days of continuous study.

While it was shown that changes in weight and venous pressure were concordant, but both varied inversely with the sodium and water excretion, a change in weight or venous pressure or sodium excretion could not be shown to precede a change in either of the other. Thus, from the data obtained, it is quite unlikely that a mere increase in intracapillary hydrostatic pressure is responsible for these changes. It could not be demonstrated that either sodium retention or in-

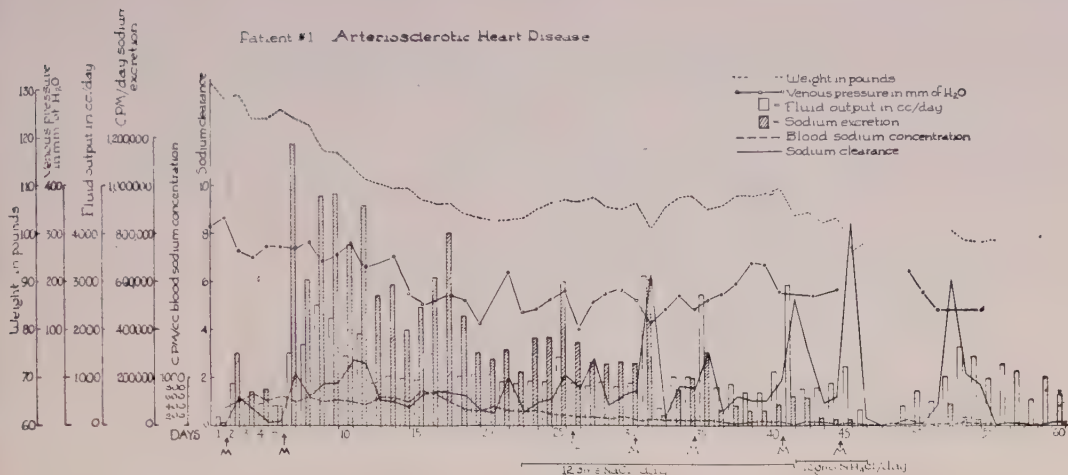


FIG. 2.

Variations in weight, venous pressure, fluid output, radiosodium (Na^{22}) excretion, blood radiosodium concentration and radiosodium clearance in a 62-year-old negro male with senile arteriosclerotic heart disease in congestive heart failure (Functional Class IV) who improved under treatment. Weight and venous pressure decreased concordantly associated with an excretion of radiosodium exceeding that of the normal subject. Addition of 12 g of NaCl per day to the diet caused a concordant increase in weight and venous pressure concurrent with a decreasing excretion of radiosodium. Intramuscular injections of Mercuhydrin (M) in 2 cc doses caused increases in radiosodium clearance, total sodium excretion and total water output. One-half of the injected radiosodium was excreted in 25 days and 64% by the end of the study (60 days). The patient was on a low sodium diet except from the 22nd to 41st day. On the 15th day K^{42} was administered for other study purposes.

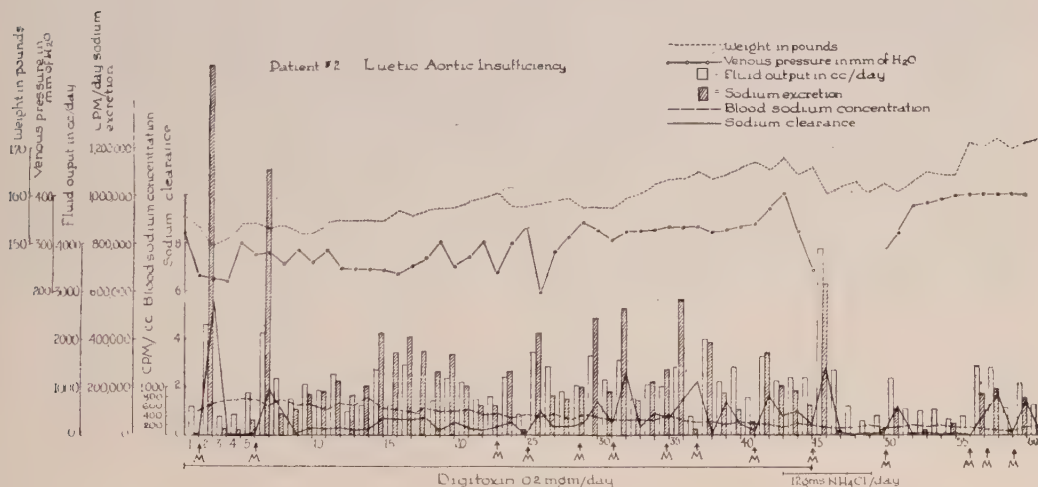


FIG. 3.

Variations in weight, venous pressure, water output, blood radiosodium (Na^{22}) concentration, radiosodium clearance and radiosodium excretion in a 46-year-old negro male with luetic aortic insufficiency admitted in congestive heart failure who decompensated further while under observation. The weight and venous pressure increased concordantly. The rate of excretion of radiosodium was less than that of the normal subject; only 42% of the injected Na^{22} was excreted in the 60 days. Intramuscular injections of 2 cc of Mercuhydrin (M) produced an increase in radiosodium excretion, blood radiosodium clearance and water output. The patient was on a low sodium diet during the entire study and digitalized during the first 45 days. On the 15th day, K^{42} was administered for other purposes.

RADIO SODIUM EXCRETION

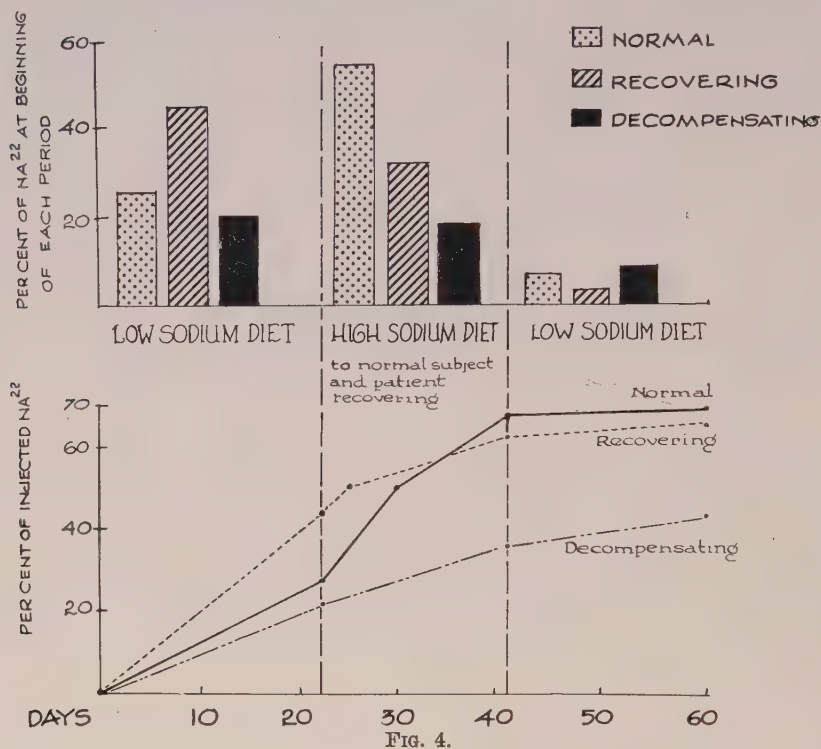


FIG. 4.

The rate of excretion of radiosodium (Na^{22}) as per cent of the total injected dose for the subjects studied. The study may be divided into 3 periods, (1) during the first 22 days the subjects were on a low sodium diet, (2) during the next 19 days the normal subject and the patient recovering from heart failure were receiving 12 g of NaCl per day, and (3) during the remaining period of the observation all 3 subjects were on a low sodium intake. The histograms show the relation of radiosodium output in the normal subject to that of the patients with heart disease, both while on a low sodium diet and while receiving 12 g of NaCl per day. These latter per cent values represent proportions of the amount of Na^{22} in the body at the onset of each of the 3 periods.

crease in venous pressure was the primary factor in edema formation or that edema predisposed to either of these factors. The actual

mechanisms of the retention of Na and water in congestive heart failure remain unknown.

Stratification of the Erythrocytes of Man by Ultracentrifuging.*

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The shape and structure of the mammalian erythrocytes have attracted the attention of many investigators.¹⁻⁴ The chief problems involved seem to center around a possible explanation for the biconcave form assumed by the erythrocytes when floating freely in the plasma, and on efforts to deduce an architecture suitable to account for the function and physiological behavior of these cells.

Beams and Hines⁵ found that it was possible to stratify the rat erythrocytes by ultracentrifugation. Similar studies dealing with the physical properties of the erythrocytes of man have been made and are herewith reported.

Material and Methods. Freshly drawn blood from the finger of an adult man was placed in the rotor of the ultracentrifuge which was subsequently sealed. After standing until the blood had clotted the centrifuge was started and operated at a pressure which delivered a centrifugal force of approximately 400,000 times gravity. After centrifuging for 20 to 30 minutes the blood was fixed in Champy's fluid for 12 hours. Paraffin sections were made of the clot which contained many erythrocytes in its meshes. These sections were bleached in potassium permanganate and oxalic acid and subsequently stained in Heidenhain's hematoxylin or Mallory's triple stain. Several other staining procedures were also tried, including supravital methods.

Results. The cells in Fig. 1 and 2 have

been stained in Heidenhain's hematoxylin. They are slightly elongated and their typical biconcave appearance seems to have been altered. The centrifugal halves of the cells stain sharply with the hematoxylin while the centripetal portion remains unstained. It is evident that the erythrocytes possess a membrane which is capable of resisting considerable mechanical distortion. Fig. 3 and 4 show erythrocytes in profile that have been stained in Heidenhain's hematoxylin. The hematoxylin-stained material has been concentrated at the centrifugal end of the cells without greatly distorting their biconcave appearance. Because these cells were free to shift their positions relative to the centrifugal field during the process of removal from the centrifuge, they are not all oriented in the same direction in the photographs.

In Fig. 5 the cells were stained in Heidenhain's hematoxylin and acid fuchsin. In the isolated cells of the lower half of Fig. 5 separate layers may be observed: a hematoxylin-staining layer at the centrifugal end, a non-staining area in the middle, and an acid fuchsin staining portion at the centripetal end. Similarly, 3 distinct layers may be observed following staining in Mallory's triple stain (Fig. 6). Here the centrifugal portion of the cells is stained orange, the middle red, and the centripetal end a light blue. The fact that they are more or less selectively stained by different dyes indicates that they are of a different chemical composition.

Discussion. The erythrocytes seem to contain at least three distinct substances which differ in their relative specific gravities and staining reactions. However, the chemical composition of these substances is at present unknown. Supravital preparations did not reveal clear evidence of stratification, although it was previously observed for the erythrocytes of the rat that the hemoglobin in some

* Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

¹ Winthrobe, M. W., *Medicine*, 1930, **9**, 195.

² Krumbhaar, E. B., *Special Cytology*, Paul B. Hoeber, New York, 1932, **2**, 555.

³ Ponder, E., *Protoplasma Monographien*, Berlin, 1934, **6**, 66.

⁴ Isaacs, R., *Handbook of Hematology*, Paul B. Hoeber, 1938, **1**, 5.

⁵ Beams, H. W., and Hines, E. B., *Anat. Rec.*, 1944, **90**, 155.

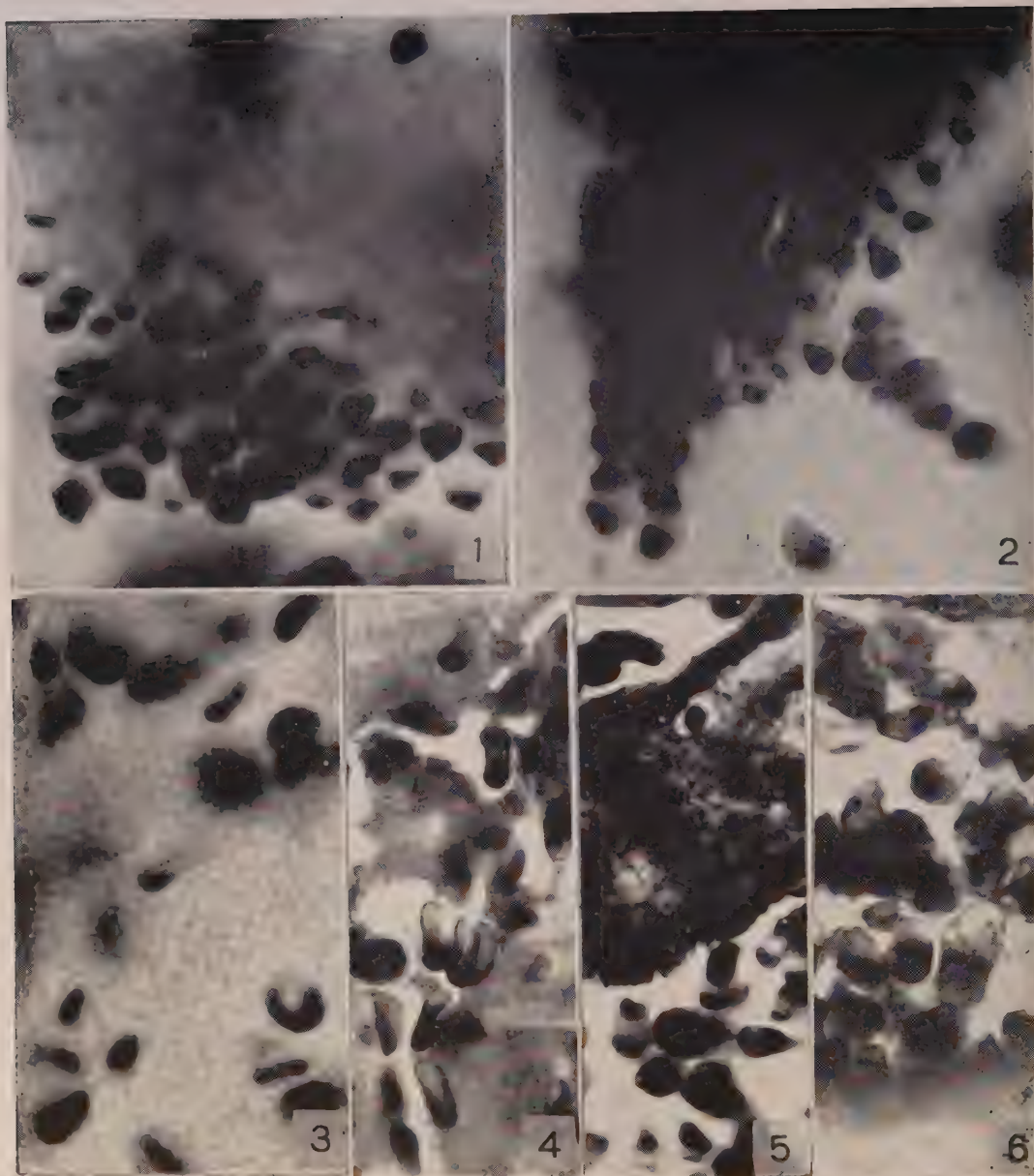


FIG. 1-6.

Centrifugal ends of cells for the most part directed toward the bottom of plate. All cells magnified approximately $1250\times$. 1 and 2. Stratified erythrocytes, hematoxylin stained. 3 and 4. Similarly treated cells in profile, normal shape evident. 5. Hematoxylin and acid fuchsin stained, 3 layers present. 6. Mallory's stained, three layers evident.

of the cells was displaced. The absence of a sharply visible stratification in the living unstained erythrocytes is not surprising because no internal structure can be seen in the normal cells with direct illumination, dark field, or with ultraviolet light.³ Although the

appearance of the stratified layers may be somewhat altered by different staining procedures, it is not likely that they represent artifacts any more than do stained structures of other cells treated in a similar way. No evidence of a stained reticulum was observed

in these studies, although we have observed it in normal uncentrifuged erythrocytes treated by other methods.⁶

Summary. Three distinct substances which differ in their relative specific gravities have

⁶ Jordan, H. E., Kindred, J. E., and Beams, H. W., *Anat. Rec.*, 1930, **46**, 139.

been stratified by high centrifugal force in the erythrocytes of man. These substances seem to differ chemically for they may be differentially stained by certain dyes. The stretching of certain of the erythrocytes by centrifugal force indicates that they possess a membrane of considerable elasticity.

16096

Influence of Epinephrine on Vasoconstrictor Action of Kidney Extracts.*

E. MYLON, F. H. HORTON AND R. P. LEVY. (Introduced by M. C. Winternitz.)

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Perfusion of rabbit's ears with saline extracts of minced kidney is usually associated with vasoconstriction. Minute amounts of histamine or histamine-like compounds present in the minced organ are thought to be responsible for this effect. When these are removed by dialysis, acetone-ether treatment and other methods involved in fractionation of the crude preparation, the vasoconstrictive action as determined with the rabbit's ear or the dog's tail, is lost.

It is reported, further, that the vasoconstrictive action of some kidney fractions as demonstrated by intravenous injection in the living animal may be absent when tested by the perfusion method.^{1,2,3} Important conclusions, it will be recalled, have been drawn from this discrepancy as determined by the two methods of testing the same extract.^{2,3} Little if any attention on the other hand has been given the reports of Schaumann,⁴ Burn and Tainter⁵ and more recently of Morton and Tainter.⁶ These authors observed that various

sympathomimetic amines are vaso-inactive when perfused through isolated peripheral blood vessels. When, however, subthreshold amounts of epinephrine are added to the perfusate, strong vasoconstriction follows.

In the light of these findings the vasoactivity of various kidney extracts has been re-examined.

As test object the vessels of the isolated rabbit's ear were selected. This is a technique devised by Pisemski and Krawkow,⁷ modified by Schlossman,⁸ Kahlson and von Werz,⁹ and further by Brandt and Katz.¹⁰ More recently it has been extensively employed by Page¹¹ who incorporated pulsating pressure. It is the general opinion that this method adequately permits measurement of vasoconstrictive substances in perfusion fluids.

Materials and Methods. In general, the experiments followed the technique described by Landis¹² and by Morton and Tainter.⁶ A constant, non-pulsating perfusing pressure

* Aided by a grant from the Commonwealth Fund.

¹ Hessel, G., *Klin. Wchnschr.*, 1938, **17**, 843.

² Braun-Menendez *et al.*, *Renal Hypertension*, translated by Dexter, L., Chas. C. Thomas, Springfield, Ill., 1946.

³ Page, T. H., and Helmer, P. M., *J. Exp. Med.*, 1940, **71**, 29, 495.

⁴ Schaumann, O., *Arch. Path. Pharmacol.*, 1928, **138**, 208.

⁵ Burn, J. H., and Tainter, M. L., *J. Physiol.*, 1931, **71**, 169.

⁶ Morton, M. C., and Tainter, M. L., *J. Physiol.*, 1940, **98**, 263.

⁷ Krawkow, N. P. *et al.*, *Z. f. die ges. Exp. Med.*, 1922, **27**, 127.

⁸ Schlossman, E., *Arch. f. Exp. Path. u. Pharmacol.*, 1927, **121**, 160.

⁹ Kahlson, G., and von Werz, R., *ibid.*, 1930, **148**, 173.

¹⁰ Brandt, G., and Katz, G., *Z. f. Klin. Med.*, 1933, **123**, 23.

¹¹ Page, T. H., *Am. Heart J.*, 1942, **23**, 336.

¹² Landis, E. M., *et al.*, *Am. J. Physiol.*, 1943, **139**, 26.

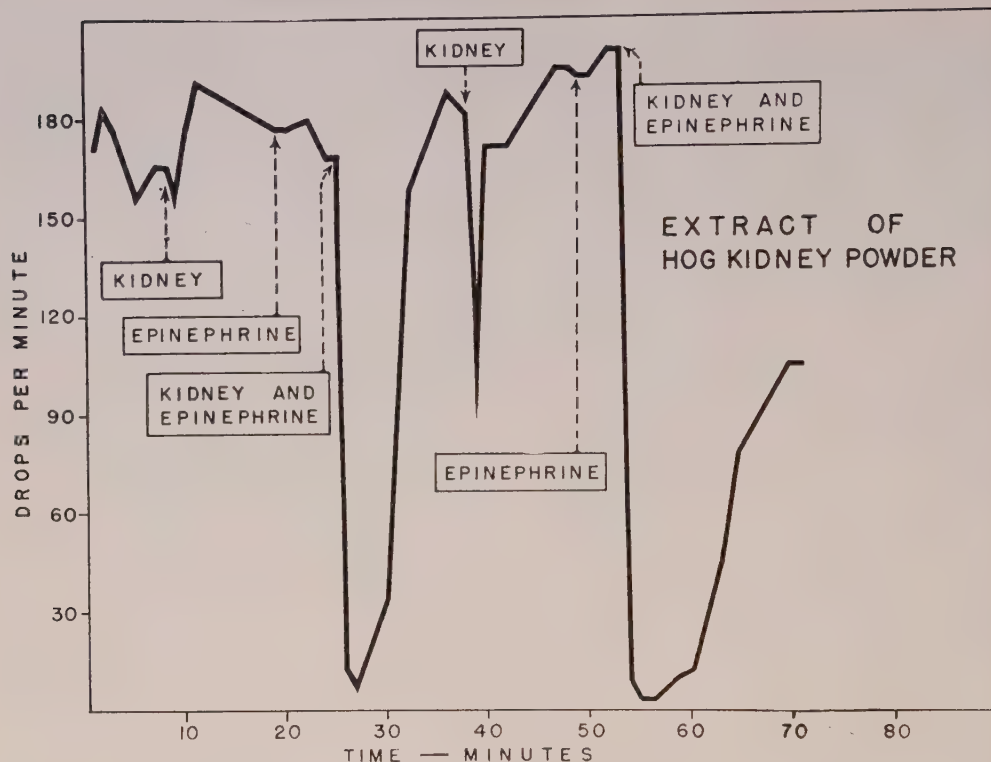


Fig. 1.

Vasoconstrictor action of a combination of an extract of hog kidney powder and epinephrine. Epinephrine amount used: 1 cc of 1:2,500,000.

was employed since Morton and Tainter⁶ did not observe any significant differences between constant and pulsating pressure as tested on the hind leg of the cat. Careful washing out of the ear with Ringer-Locke solution preceded each injection of test material. Epinephrine (Parke, Davis 1:1000) was diluted with distilled water and finally 0.5 cc, 1.0 cc or 1.5 cc of this 1:2,500,000 or 1:5,000,000 solution was made up to 8 cc with Ringer-Locke. The subthreshold dose was determined for each ear and was found to range between .2 and .6 μ g. When a mixture of epinephrine and a kidney extract was to be tested, an appropriate amount of Ringer-Locke solution was replaced by kidney extract, the total volume remaining 8 cc.

Hog Kidney Fractions. The simplest of these preparations resulted from the extraction of the dry powder secured by acetone-ether treatment of fresh minced hog kidney. Twenty grams of the powder were extracted with approximately 150 cc of 2% saline; this

suspension was then centrifuged and the supernatant dialyzed.

A second preparation made in this laboratory in 1939¹³ resulted from the further treatment of the above saline extract with mono- and dipotassium phosphate and then with 10% sodium chloride at pH 4.0.¹⁴ On intravenous injection 3.6 mg of the resulting protein raised the blood pressure of a 10 kilo dog 30-40 mm of Hg.

A third preparation was made recently by a different method. Aqueous extracts of hog kidney brought to pH 2.9 with 10% trichloroacetic acid were filtered, neutralized and fractionated with ammonium-sulfate and alcohol. The procedure is similar to one described for the preparation of renin.¹⁵ 0.36 mg of the

¹³ Winternitz, M. C., Mylon, E., Waters, L. L., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **12**, 623.

¹⁴ Helmer, P. M., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 757.

¹⁵ Katz, Y. I., and Goldblatt, H., *J. Exp. Med.*, 1943, **78**, 67.

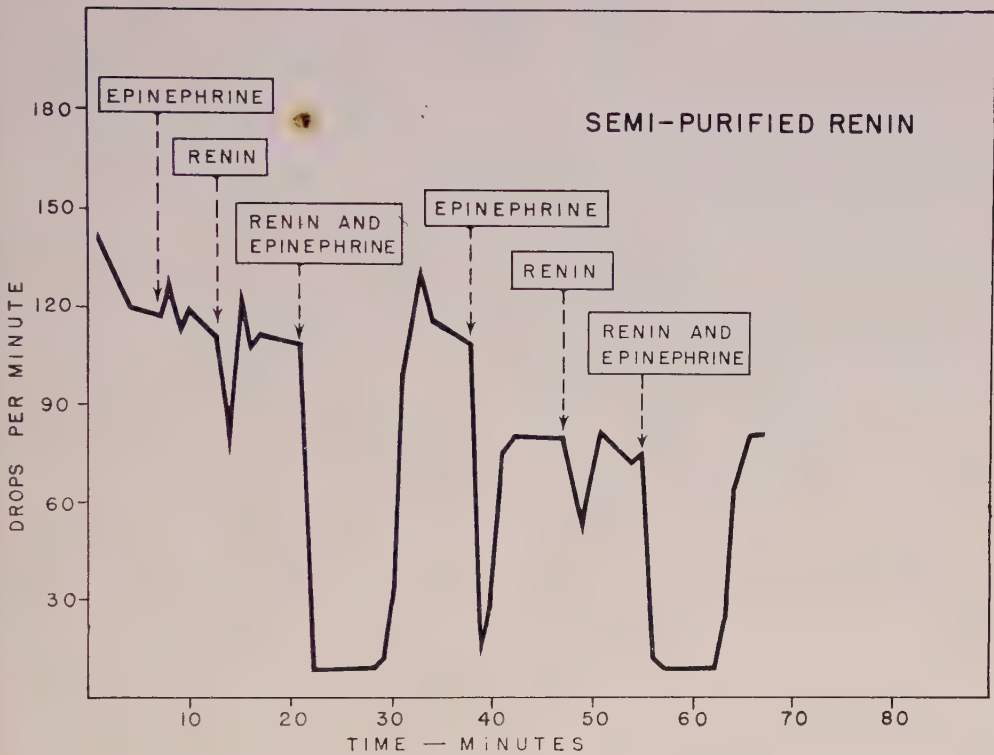


FIG. 2.

Vasoconstrictor action of a combination of semi-purified renin and epinephrine. Epinephrine amount used: 1 cc of 1:2,500,000.

resulting lyophilized protein mixture injected intravenously raised the blood pressure of a 10 kilo dog 30-40 mm of Hg.

Results. The saline extract of the dry powder produced no significant effect on the flow of the perfusate through the rabbit's ear in 10 trials. When combined with a quantity of epinephrine, in itself without effect on the perfused vessels, marked vasoconstriction followed. A typical experiment is recorded in Fig. 1.

A short constrictor effect was occasionally encountered on the second or third injection of amounts of epinephrine that were "sub-threshold" on the first injection. This contrasted with the prolonged constriction that followed the combination of epinephrine and renin.

The second preparation gave equivocal results. In itself inactive on perfusion, vasoconstriction followed with addition of a sub-threshold quantity of epinephrine in 16 of the 26 trials. The preparation was found to be

only relatively soluble, as might have been anticipated after years of storage and this is thought to be associated with the inconstant results secured.

The findings were uniform with the recently prepared and quite soluble third preparation. No vasoconstriction followed when it was added to the isolated rabbit ear perfusate in many trials. When, however, it was supplemented with the above described minimal amounts of epinephrine marked vasoconstriction invariably resulted. This is illustrated in Fig. 2.

Summary. Protein fractions of hog kidney, in themselves inactive on perfusion, become strongly vasoconstrictive when supplemented with traces of epinephrine. While a similar influence of minute quantities of epinephrine on vasoconstriction is well known to occur with some sympathomimetic amines, insufficient information is at hand to clarify the pharmacological nature of the observed synergistic action.

Influence of Epinephrine on Vasoconstrictive Action of Organ Extracts.*

E. MYLON, F. H. HORTON AND R. P. LEVY. (Introduced by M. C. Winternitz.)

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Epinephrine quantitatively insufficient in itself to have any constrictor effect has been observed to activate various otherwise inert fractions of kidney extract as demonstrated by constriction of the vessels of the perfused rabbit's ear.¹ It has been shown that this effect is neither species nor organ specific. This evidence will now be presented together with such facts as have been secured related to the character of the agent in the tissue extracts.

Organ extracts of hog, dog, and rabbit including liver and heart as well as kidney have been prepared in the following several ways:

(1) Approximately 200 g of minced organ were thoroughly mixed with equal amounts of saline, kept at 5°C for 24 hours prior to removing the precipitate by centrifugation and dialysis of the supernatant fluid.

(2) Twenty g of the dry powder residue after acetone-ether extraction of minced organ, were treated with approximately 150 cc of 2% saline and centrifuged to secure the supernatant for dialysis.

(3) Many methods of fractionating the above more crude extracts were utilized. Only the one that yielded a product of greater interest is detailed. Approximately 5 kg of fresh hog liver were minced. The following procedures were carried out at 5°C: Extraction with 10 liters of distilled water for 24 hours and filtration through several layers of gauze; the careful adjustment of the filtrate to pH 2.9 by slow addition of 10% trichloroacetic acid. (The final concentration of the trichloroacetic acid was 1.4%.) Removal of the precipitate through fluted filter paper (Whatman No. 2) after 6 hours. The filtrate was

then fractionated with ammonium sulphate, the precipitates redissolved and dialyzed, re-fractionated with ammonium sulphate and alcohol, in general following the procedure described for the preparation of renin from kidney.² The obtained organ fractions are of protein nature and differ from the dialysates prepared by Cannon and Lissak,³ the alcohol extracts used by v. Euler⁴ and the preparations made by Raab and Humphreys.⁵

Results. No significant vasoconstriction resulted from the perfusion of the rabbit's ear with either the dialyzed saline extracts of hog heart or liver or with the saline preparation of the powders resulting from acetone-ether treatment of any of the three organs of the several animal species. Even addition of minimal quantities of epinephrine had reasonably little effect as supplement to the saline extract of minced organ. This latter finding contrasted with the result of similar addition of epinephrine to the saline preparation derived from treatment of organ-powder after acetone-ether extraction. Vasoconstriction was always evident and as a rule it was marked. This is illustrated in Fig. 1.

Marked vasoconstrictor effects resulted from the addition of epinephrine in minimal amounts to the last fraction of the liver designated as IV A. A typical experiment is illustrated in Fig. 2.

Throughout this study attention was directed to the ratio of the nitrogen content of the perfusate to the activity curve. The trend had been downward and with Fraction IV A reached the new low. Two cubic centimeters

² Katz, Y. J., and Goldblatt, H., *J. Exp. Med.*, 1943, **67**, 78.

³ Cannon, W. B., and Lissak, K., *Am. J. Physiol.*, 1939, **125**, 765.

⁴ v. Euler, U. S., *Nature*, **157**, 369, March 23, 1946.

⁵ Raab, W., and Humphreys, R. J., *Am. J. Physiol.*, 1944, **148**, 460.

* Aided by a grant from the Commonwealth Fund.

¹ Mylon, E., Horton, F. H., and Levy, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 375.

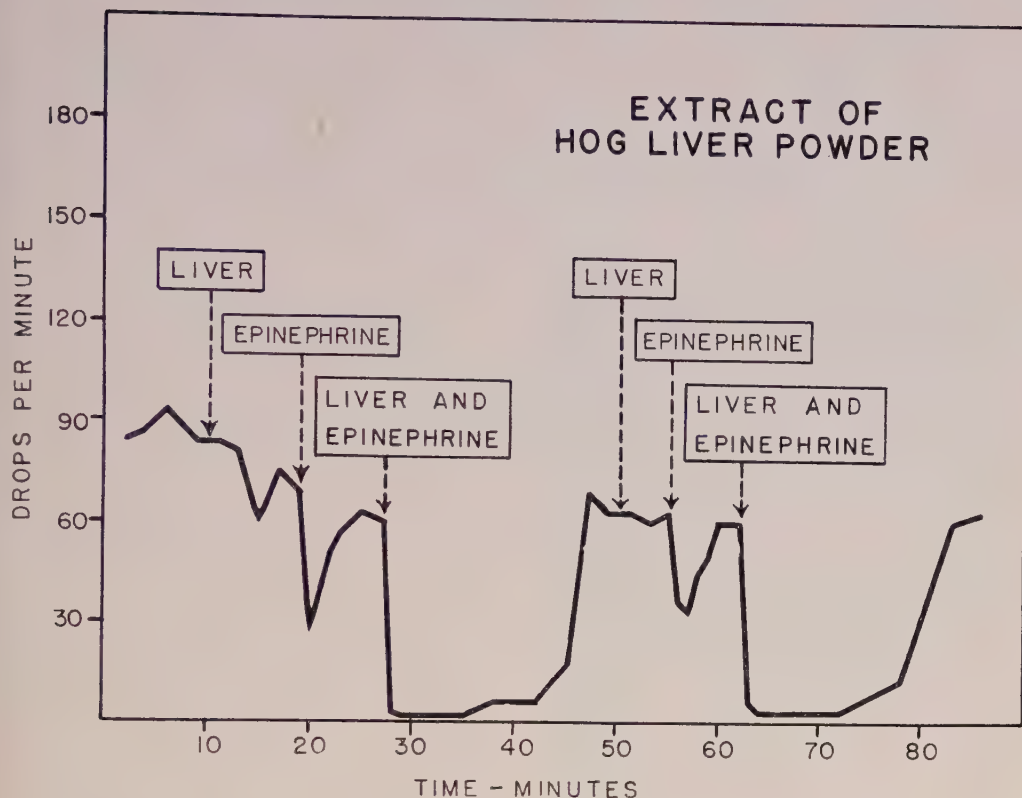


FIG. 1.

Vasoconstrictor action of a combination of an extract of hog liver powder and epinephrine. The epinephrine amount used was 1 cc of 1:2,500,000. It should be added that at least 5 times the amount of epinephrine alone is required to produce an equipressor effect.

of the extract with high activity had a total content of only 0.8 mg of protein.

To corroborate this result in part, 2 more lots of liver were processed after the same fashion detailed above. Despite rigid adherence to the prescribed method, the fractions of the two new lots with highest activity when supplemented with epinephrine were not the same. This is shown in Table I that represents 36 experiments with the various fractions of lots 2 and 3.

Analysis of Fraction IV A. This fraction

was lyophilized and no reduction of its vasoconstrictor activity resulted. Samples of the lyophilized fraction, a fluffy, light brown powder, were analyzed with the following results:

1. *Qualitative Tests:* The usual qualitative tests for protein were positive. 95% of the nitrogen was amino N. There was a positive test for sulfur.

2. *Quantitative Analysis:* All elements reported were calculated on a moisture and ash free basis.

%H	C	N	S	P*
6.98	49.18	15.18	0.95	0.06

* The phosphorus probably represents an impurity in the ash.

Discussion. Protein fractions of several organs of various species become vasoconstrictive when supplemented with subthreshold quantities of epinephrine just as such addition is known to effect otherwise inactive sympathomimetic amines.⁶ Whether this activation

TABLE I.

Activity	Fractions	Protein range, mg/cc	Lot No.
None	3	—	2
Moderate	10	0.12- 5.6	2
Marked	5	2.6 -13.2	2
None	4	0.5 - 1.7	3
Moderate	3	0.8 - 2.4	3
Marked	6	0.4 - 4.7	3

⁶ Morton, M. C., and Tainter, M. L., *J. Physiol.*, 1940, **98**, 264.

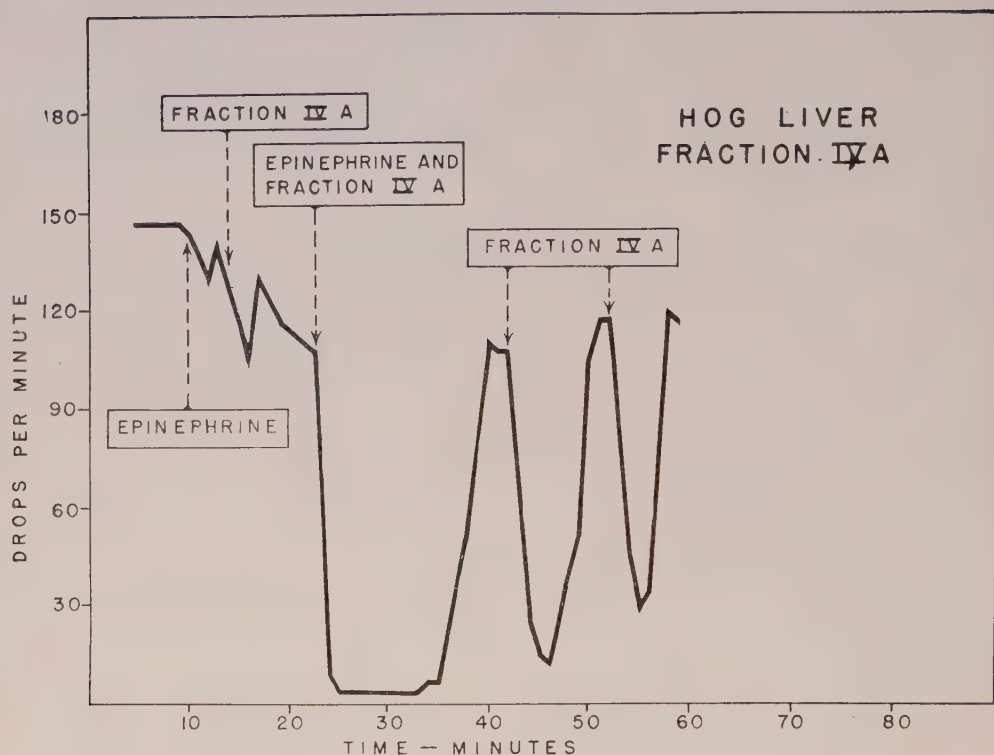


FIG. 2.

Vasoconstrictor action of a combination of the hog liver fraction IVA and epinephrine. Epinephrine amount used: 1:2,500,000. Note the slight constrictor effect of fraction IVA alone, and the marked constriction subsequent to the injection of the combination of fraction IVA and epinephrine. This marked constriction never occurred prior to the injection of the combination. No explanation is offered for this effect.

indicates a potentiation of epinephrine or a stimulation of effector cells normally responding to this compound remains unanswered.

Varying amounts of phospholipid were removed from the protein fraction by the different methods employed for separation. This may be associated with the change of the vasoconstrictor effect on blood vessels.

Summary. Many protein fractions isolated from kidney, liver and heart of hog, and from kidney and liver of dog and rabbit, inactive when perfused alone through the rabbit's ear, become vasoconstrictive at varying degrees

when combined with subthreshold quantities of epinephrine.

In contrast, dialyzed saline extracts of fresh minced organs were inactive with and without addition of epinephrine. After freezing and thawing or prolonged dialyzation against distilled water epinephrine addition resulted in some activity. 0.8 mg of protein or protein mixture secured by continued fractionation of hog liver and combined with subthreshold amounts of epinephrine, produced marked and long lasting vasoconstrictions.

A Machine for Shell-Freezing Biological Materials in Small Glass Ampoules.

JOEL WARREN. (Introduced by J. E. Smadel.)

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The increasing use of desiccation *in vacuo* from the frozen state as a method for preserving biological materials has created the need for equipment which will shell-freeze small amounts of fluid in a few ampoules without undue effort and expense. The machine described below is suitable for this purpose. It eliminates the irregularities in thickness of shell which result from rotation of ampoules by hand and the prohibitive cost of a single small run in one of the shelling machines designed for large scale production.¹

A cylindrical block, or rotor, of duraluminum, 4 inches deep and 5 inches in diameter, constitutes the main portion of the freezing unit (Fig. 1 and 2). Eight drilled holes which have a diameter of 11/16 inches for a depth of 2½ inches provide the spaces into which the ampoules fit with a minimum of play. The holes penetrate the entire block to facilitate cooling, but are reduced in diameter to ¼ inches throughout the terminal 2½ inches of their depth, the place at which the diameters change, forms a shoulder upon which the ampoule rests. A small electric motor turns the rotor at 35 r.p.m. through a reducing gear, the shaft of which is directly attached to the duraluminum block by means of a hub and set-screw. The freezing mixture of alcohol and crushed "dry ice" is placed beneath the rotor in a pan in such a manner that only a small segment of the circumference of the rotor is submerged to a depth of no more than ½ inches at its deepest point. The rotor and motor are mounted on a wedge-shaped base so that the outer surface of the rotor has an angle of about 5° with the surface of the chilled liquid.

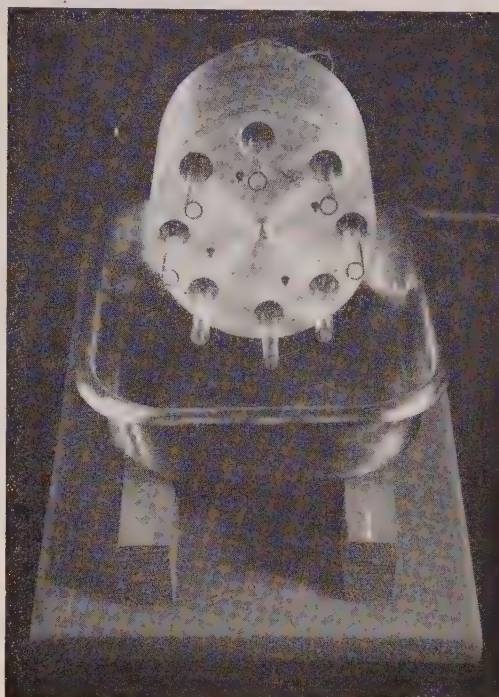


FIG. 1.

A machine for shell-freezing biological materials in small glass ampoules.

In operation, the rotor is first chilled by rotating for five minutes in the freezing bath and the cotton stoppered ampoules are then rapidly inserted in the holes. Approximately one minute is required to freeze an ampoule containing 3 cc of fluid. Approximately 400 ampoules can be processed in an hour with the apparatus illustrated. The unit is particularly useful for freezing small lots, such as one-half to 3 dozen ampoules; for this about 3 to 4 pounds of "dry ice" are required.

Summary. An apparatus is described for the rapid shell-freezing of fluids in small containers preparatory to drying from the frozen state (lyophilization).

¹ Strumia, M. M., and McGraw, J. J., *J. Lab. and Clin. Med.*, 1943, **28**, 1140.

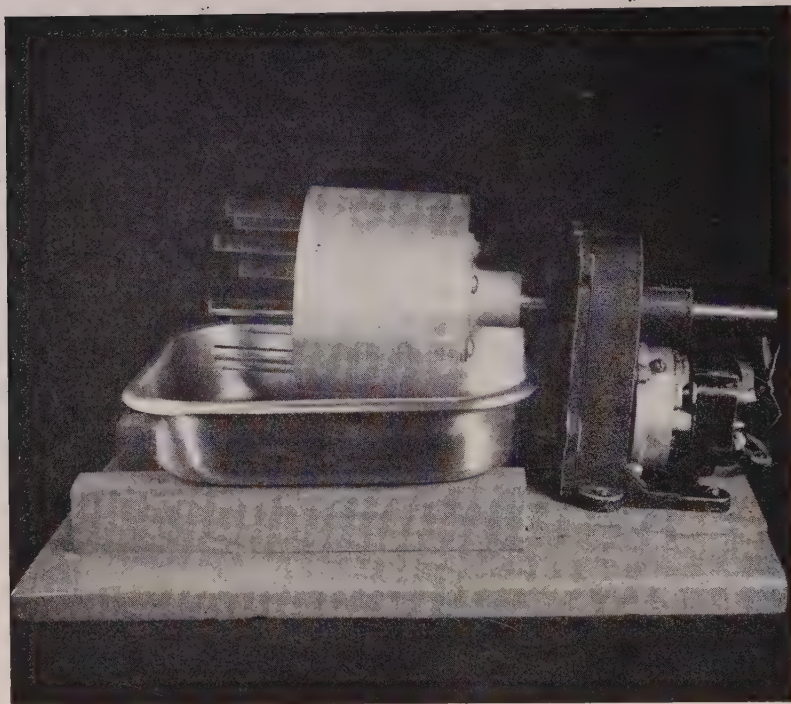


FIG. 2.
A machine for shell-freezing biological materials in small glass ampoules.

16099

Production of Fatty Infiltration of Liver in Rats by Alcohol in Spite of Adequate Diet.

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The part played by alcohol in liver cell injury and cirrhosis has not been conclusively established. Prior to the recognition of the influence of dietary factors in liver injury, many observations were made in an attempt to ascertain the solution to this question. Moon¹ summarized and critically evaluated these reports, concluding that there was no definite evidence that alcohol itself produced liver cell injury. At the time of Moon's review, 1934, the dietary factor was not given any consideration, and its significance was not

apparently recognized. Lamson and Wing² showed that the combination of alcohol with carbon tetrachloride increased the severity and incidence of liver cell injury and cirrhosis. This observation was evidently acceptable to both sides of the question as a compromise and seemed to constitute the basis for prevailing opinion on the subject until recent years. Following the elucidation of the dietary factor in the problem of liver cell injury,^{3,4,5}

¹ Moon, V. H., *Arch. Path.*, 1934, **18**, 381.

² Lamson, P. D., and Wing, R., *J. Pharm. and Exp. Therap.*, 1926, **29**, 191.

³ Allen, F. N., Bowie, D. J., McLeod, J. J. R., and Robinson, W. L., *Brit. J. Exp. Path.*, 1924, **5**, 75.

⁴ Best, C. H., and Huntsman, M. E., *J. Physiol.*, 1935, **83**, 255.

and the recognition of certain deficiency syndromes in the chronic alcoholic patient,^{6,7} there has been a progressive tendency to attribute all of the organic changes in alcoholism to the supposed universal dietary deficiency.⁸

The observations of Connor,⁹ however, are in opposition to this and should be noted. In rabbits, he was able to produce fatty infiltration, liver cell atrophy, necrosis of liver cells, and fibrosis in variable degree by daily administration of alcohol, even though a diet with high casein content and an adequate amount of other known required factors were provided. The use of the rabbit, an animal in which perilobular fibrosis and parasitic disease of the liver are common findings, the fact that the animals did not take an adequate amount of the diet, and the lack of control animals, make it difficult to assess fully the findings in Connor's experiment.

The problem has therefore been undertaken in rats. Adult female rats of the Sprague-Dawley strain were used. Four series were employed:

- Group I—Low casein diet,* and alcohol,
- Group II—High casein diet, and alcohol,
- Group III—Low casein diet, no alcohol, and
- Group IV—High casein diet, no alcohol.

The administration of alcohol caused a reduction of food intake. The amount of food

⁵ Du Vigneaud, V., *Biol. Symposia*, 1941, **5**, 234.

⁶ Alexander, Leo, *Am. J. Path.*, 1940, **16**, 61.

⁷ Goodheart, R., and Jolliffe, N., *Am. Heart J.*, 1938, **15**, 569.

⁸ Wright, A. W., *Arch. Path.*, 1941, **32**, 670.

⁹ Connor, C. L., *Arch. Path.*, 1940, **30**, 165.

* The composition of the diets¹⁰ employed was:

	Low casein	High casein
Lard	40%	40%
Agar	2	2
Starch	48	28
Salt mixture	48	28
Osborne, Mendel	5	5
Casein	5	25

Each animal was given daily one drop of Vit Penta furnished through the courtesy of Hoffmann-La Roche), each 0.6 cc of which contains 5000 USP units of vitamin A, 1 mg of thiamine HCl, 1 mg of riboflavine, 50 mg of ascorbic acid, 1000 USP units of activated ergosterol, and 2 mg of niacinamide.

¹⁰ Tidwell, H. C., and Treadwell, C. R., *J. Biol. Chem.*, 1946, **162**, 155.

administered was therefore placed under control by allowing Groups I and II to eat *ad libitum*, and the amount taken was determined by weighing the food containers. The amount of food given to Group III and IV was the same as that eaten by the corresponding group taking alcohol, and on the preceding day. Alcohol was administered daily by stomach tube, the amount of 1.5 cc of 25% alcohol per 100 g, and 10% alcohol was given as the only drinking water. Approximately 0.6 cc of absolute alcohol per 100 g of body weight was taken daily by these animals. The regime was continued for about 2 months, and at the end of this time the animals were autopsied and histological study of the livers and other organs made.

Results. The rats receiving alcohol became obviously intoxicated and not infrequently were comatose for short periods following the administration of alcohol. These animals ate poorly. The groups receiving no alcohol continued to exhibit good appetite throughout, although the animals receiving low casein diet later became slightly less inclined to eat their food.

The weight declined in all groups. Fig. 1 and 2 show the decrease for each animal.

Fatty livers were found in every animal of Groups I, II and III. No fatty change was found in Group IV. The gross and histological findings were characteristic of this degenerative lesion. The liver was increased moderately in weight and was somewhat yellowish and friable. Histological study of hematoxylin-eosin and Sudan stained sections revealed fatty deposition in the liver cells and was moderate or marked, predominantly the latter. No evidence of cirrhosis was found.

Discussion. In these experiments, the effect of emaciation from quantitative nutritional lack has been eliminated by providing the same amount of food to the corresponding control and alcohol groups. The weight curves further establish this point, since the group receiving alcohol and adequate diet, showed slightly less weight decline than those receiving no alcohol and quantitatively adequate diet. Thus any liver cell injury might well

WEIGHT CURVES ON GROUPS I AND III

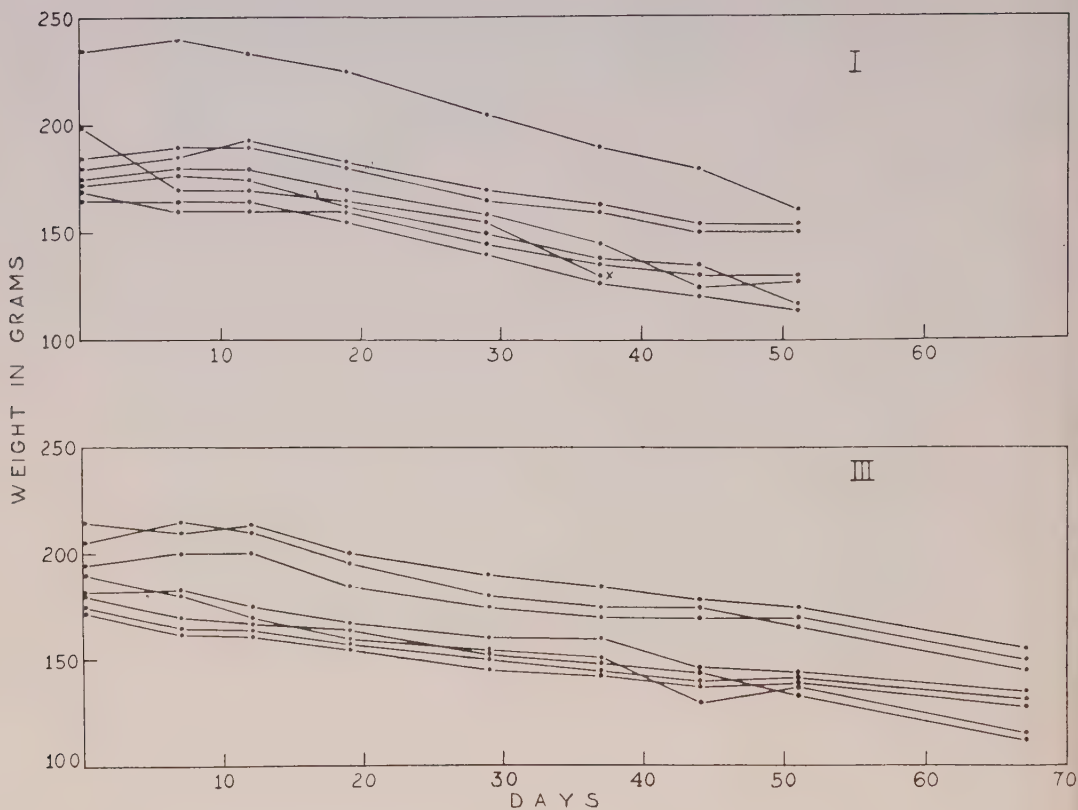


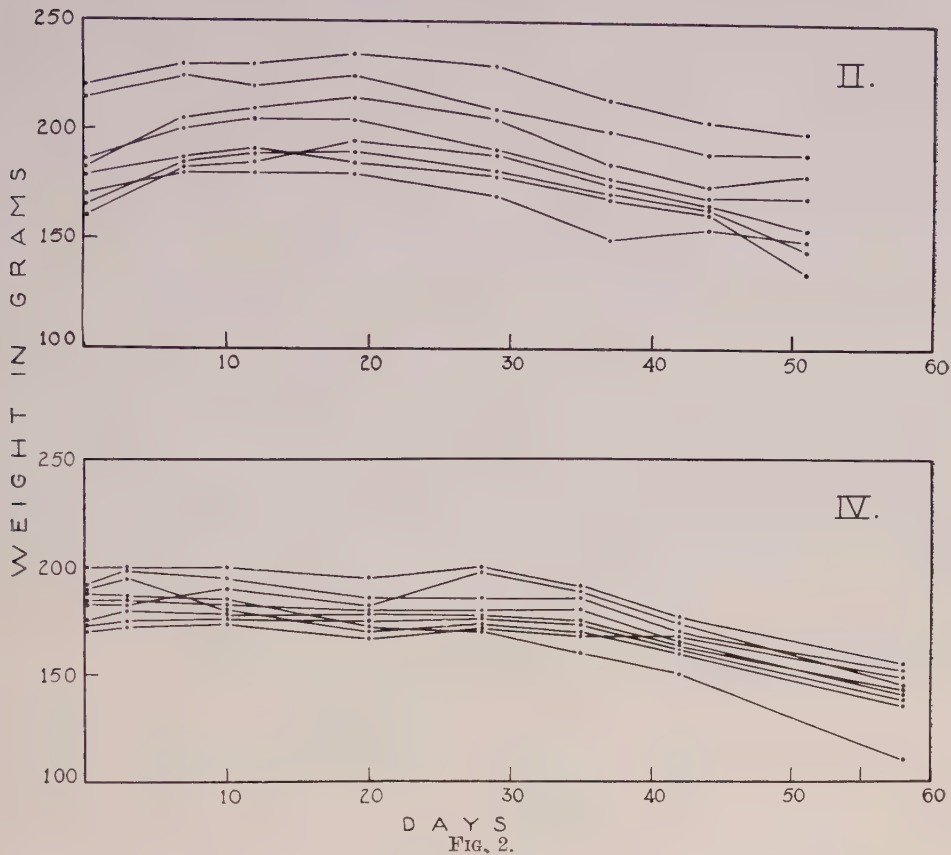
Fig. 1.

be attributed to the only apparent variable, namely the presence or absence of alcohol. The fact that in Group II, receiving high casein diet and alcohol, fatty infiltration of the liver occurred in all animals, would seem therefore to indicate that alcohol itself does produce liver cell injury, and in this instance, fatty infiltration. There was no apparent difference in the incidence or severity of fatty degeneration nor is there any histological difference in the character of the lesion in the animals receiving alcohol and high casein diet, as compared to those receiving low casein diet with or without alcohol. The duration of the period of observation was too short to determine the occurrence of cirrhosis in these animals. It remains to be shown whether the liver cell injury produced by alcohol is due to a direct toxic action, to the effect of the degradation products of alcohol,

or whether alcohol interferes with the chemical processes normally called into play in the metabolism of fat.

Summary. Rats were given alcohol daily and either a high or low casein diet. Control series received similar diets and no alcohol. The amount of food intake in control groups was regulated according to the amount taken by the rats receiving alcohol. The effect of total food intake in those receiving alcohol as compared with those not receiving alcohol was further observed by weight curves. Fatty infiltration of the liver occurred in every animal receiving alcohol and low casein diet, alcohol and high casein diet, and in all those receiving low casein diet and no alcohol. None of the animals receiving the same volume of the high casein diet and no alcohol had fatty infiltration of the liver. It is therefore concluded that alcohol exerts an effect

WEIGHT CURVES ON GROUPS II AND IV

DAYS
Fig. 2.

which permits accumulation of fat within the liver cells, and that this effect operates separately from that of extrinsic deficiency of lipotropic factors.

16100

Complement-Fixation in Experimental and Human Poliomyelitis.*

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Although many attempts have been made to find a simple immunological means of studying poliomyelitis, only the neutralization reaction has yielded consistent and reliable results. Possible reasons for the lack of success of precipitin and complement-fixation

tests have been discussed by several authors.¹ The possibilities include (a) low reactivity or avidity of the neutralizing antibodies, (b) small size of the virus particle, and (c) insufficient concentration of virus in the antigen

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Schultz, E. W., Gebhardt, L. P., and Bullock, L. T., *J. Immunol.*, 1931, **21**, 171; Raffel S., and Schultz, E. W., *J. Immunol.*, 1940, **30**, 256.

TABLE I.
Complement-fixation with Concentrated Lansing Virus and Sera from Immune Rats.*

Immunization history	Rat No.	Serum 1:				Serum control	Titer (units per ml)
		0	2	4	8		
Group a, vaccinated rats which developed "polio" on challenge, but survived with residual paralysis.	66	4	4	4	3	0	80
	67	4	2	±	±	0	20
	94	4	4	4	2	0	80
Group b, vaccinated rats which proved resistant to subsequent challenge	90	2	2	±	±	0	20
	91	1	1		0	0	+
	92	±	0	0	0	0	0
Group c, same as group b, vaccinated with a different preparation of formalized virus	63		4	4	4	0	>80
	64		4	4	4	±	>40
	95		4	2	4	0	>80
	100			4	4	0	>80
	102		4	4	4	4	>80
	107			4	4	4	>80
	113		4	4	4	4	>80
	116		4	4	4	4	>80

Groups a and c. Injected with 10^{-5} g formalized virus nitrogen, Preparation 91-2-4,³ as follows: 2.5×10^{-6} g intradermally on 12/16/46, 5×10^{-6} g intramuscularly on 12/23/46, and 2.5×10^{-6} g intradermally on 12/30/46. Challenged on 1/6/47. Bled 1/21/47.

Group b. Injected with 3×10^{-6} g formalized virus nitrogen, Preparation 87³ as follows: 2×10^{-3} g on 8/15/46 and 10^{-6} g on 8/20/46 intraperitoneally. Challenged 8/24/46. Rechallenged 9/27/46. Reinjected with 0.5 cc of 20% crude virus suspension twice weekly from 10/12/46 to 1/2/47. Bled 1/21/47.

* Virus preparation 91-2-4³ at 2×10^{-5} g nitrogen per ml used as antigen; antigen control with one unit complement = 0, with $\frac{1}{2}$ unit complement = 2, and with no complement = 4.

preparations used.

Recent work on the concentration of the Lansing strain of poliomyelitis virus² has provided more concentrated virus preparations than those previously employed in immunological tests. In a previous paper³ the immunization of cotton rats with formalized preparations of highly concentrated Lansing virus and their protection against the intracerebral injection of large doses of active virus was reported. If the high degree of immunity produced in many of these animals was due to a high titer of humoral antibodies, it seemed possible by the use of concentrated virus as antigen that positive precipitin or complement-fixation tests could be demonstrated in such sera. Precipitin tests were attempted but proved negative. Complement-fixation tests, on the contrary, proved definitely positive.

The results obtained with sera from 2 groups of rats that had been injected with formalized virus and had proved refractory to relatively large amounts of active virus,

as well as with sera from 3 immunized rats which developed poliomyelitis on subsequent challenge, are given in Table I. The complement-fixation tests were made as follows: The immune sera were incubated at 56°C for 20 minutes to destroy the complement present and mixed with saline to give the dilutions used. A 0.1 ml volume of each dilution was then distributed in small test tubes, to which was added 0.2 ml of a dilution of guinea pig complement containing 2 units, 0.6 ml of saline, and 0.1 ml of a 2-cycle virus preparation containing 10^{-5} g nitrogen per ml. Preliminary tests showed this concentration of virus to be devoid of anticomplementary properties when tested with fractional units of complement. Controls without antigen were set up for the entire range of dilutions of the sera. Controls for possible anticomplementary action of the antigen were also set up along with each set of tests. The tubes were shaken and incubated at 4°C overnight. To a 4% suspension of sheep red blood cells was added an equal volume of solution containing eight units of hemolysin per ml and 0.5 ml of this suspension of sensitized cells was added to each test tube. The tubes were shaken, in-

² Loring, H. S., and Schwerdt, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 291.

³ Loring, H. S., Schwerdt, C. E., Lawrence, N., and Anderson, J. C., *Science*, 1947, **106**, 104.

cubated at 37°C for one hour, set in the refrigerator for 2 hours or slightly more to permit settling of cells, and read. The last tube showing 50% lysis was taken to determine the titer. Titer is expressed in these experiments as the number of units of complement-fixing antibody contained in one ml of undiluted serum. It is worthy of emphasis that in carrying out these tests the following precautions were observed: (a) The tubes were incubated at low temperature for eighteen hours to assure maximum reaction and minimal deterioration of complement. (b) The antigen was tested for possible anti-complementary properties by incubation with fractional units of complement in advance of and simultaneously with each group of tests. (c) A control for anticomplementary properties of each serum was made throughout the dilution range simultaneously with the actual test.

The data presented in Table I show that complement-fixing antibodies against active virus were present to a variable degree after immunization with formaldehyde-inactivated virus. As both the method of vaccination and the elapsed time before blood was drawn varied in these experiments, it was reasonable to expect the type of variation found. When blood was drawn about three weeks after the last injection of vaccine in the animals which were refractory to infection, Group c, uniformly high titers of >80 were found. Blood from vaccinated animals which developed poliomyelitis but recovered with some residual signs of the disease, Group a, also showed similar high titers. Samples taken five months after immunization, however, (Group b) gave relatively low titers although these animals had been given several injections of crude 20% infected-brain extract a few weeks previously.

As the vaccine used for immunization in the above mentioned experiments had been obtained from a virus preparation that may have contained some normal rat tissue proteins, the positive results could be explained by immunization to the normal tissue components as well as by immunization to virus. In order to study this question, the complement-fixing

TABLE II. Complement-fixation of Immune Sera with Concentrated Virus and Normal Tissue Antigen.

[illegible]

* Injected with 10^{-5} g formalized virus nitrogen. Preparation 91-2, 4.3 as follows: 2.5×10^{-6} g intradermally on 1/24/47; 5×10^{-6} intramuscularly on 1/31/47 and 2.5×10^{-6} g intradermally on 2/7/47. Challenged on 2/17/47. Bled 3/10/47.

+ Same group of rats as Group c, Table I. Bled 4/8/47.

Virus preparation 91-2.43 used at 10.5 g nitrogen per ml; preparation 91-2.43 used at 10.5 g nitrogen per ml³ and at 10.6 g nitrogen per ml as indicated.

titer of each serum was determined with a similarly concentrated preparation of normal cotton rat brain and spinal cord in comparison with that of concentrated virus.

A total of 257 g of brains and spinal cords from normal cotton rats were processed by the procedure used for the preparation of the virus concentrate.² After 2 ultracentrifugal cycles of purification, the high molecular weight sediments obtained contained 0.85 mg of nitrogen. The yield of nitrogen found in this experiment was relatively higher than those obtained from normal tissue after 3 or 4 cycles of purification as previously reported. This normal constituent was used in the complement-fixation tests at the same concentration as that used for the virus preparation, namely 10^{-5} g nitrogen per ml, and also at one-tenth this concentration.

The results of this experiment are summarized in Table II. It may be seen that a sample of pooled immune serum from 6 immunized and refractory rats fixed complement when either the concentrated virus or the preparation from normal rat tissue was used as antigen. In all the tests made, however, the titer for virus (160 to 1280) was significantly higher than that for the normal preparation at either the same concentration, (titers of 80 to 160) or at one-tenth the concentration (titers of + to 40). Because of the difference in titers with the 2 antigens, the results suggest that complement-fixation in the presence of virus was due primarily to the reaction of antibody with virus rather than to a reaction of antibody with a normal component present in the virus preparation. Another possible explanation for positive complement-fixation with the antigen from normal tissue, in addition to that mentioned above, is that poliomyelitis virus produced in cotton rats may be related antigenically to certain normal brain tissue proteins. The latter phenomenon would be similar to that observed with several infectious agents and certain of the normal antigens of the host.^{4,5,6}

Experiments were conducted with sera from normal rats to determine if antibodies were present which would fix complement in the presence of either virus or the normal tissue preparation. These results are summarized in Table III. It may be seen that complement-fixation occurred with most of the sera tested with virus as antigen in animals either 5 or 8 weeks old. Less consistent results and in general lower titers were found with the "normal" antigen. Several instances are recorded where positive tests were found with virus, but no antibodies to the "normal" antigen could be detected. These results are similar to those found by Casals and Palacios⁷ and by Kidd and Friedewald⁸ with non-specific antibodies occurring in normal serum. In the latter instances it was reported that the non-specific antibody could be eliminated by heating the serum to a higher temperature than that usually employed to destroy complement. It was of interest to determine if the reaction with the virus preparation and the "normal" antibody could be similarly eliminated. Two normal sera which fixed complement in the presence of virus after they had been heated at 56°C for 20 min. were heated for an additional 20 min. at 60°C and again tested with the virus concentrate as antigen. In both cases negative tests were now found indicating that the normal antibody had been eliminated by this method.

The same procedure was next applied to the 5 immune sera shown in Table II which had fixed complement in the presence of either virus or the "normal" antigen. After they had been heated 20 min. at 60°C, they were again tested with virus and with the "normal" antigen. In 4 of the 6 samples the reaction with the "normal" antigen was eliminated whereas positive complement fixation (titer of 20 to 80) was still found with the virus concentrate.

A consideration of the results obtained in all the experiments mentioned seemed to justify the conclusion that complement fixa-

⁴ Duran-Reynals, F., *Yale J. Biol. and Med.*, 1940, **13**, 61.

⁵ Chambers, L. A., and Henle, W., *Am. J. Path.*, 1941, **17**, 442.

⁶ Knight, C. A., *J. Exp. Med.*, 1946, **83**, 281.

⁷ Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

⁸ Kidd, J. G., and Friedewald, W. F., *J. Exp. Med.*, 1942, **76**, 543.

TABLE III.
Complement-fixation of Normal Rat Serum with Virus and with "Normal" Antigen.

History of animals	Virus preparation	Serum dilution and degree of complement-fixation with antigen shown																Titer for virus	Titer for "Normal" virus		
		Virus; Serum 1:				"Normal";* Serum 1:				Serum control											
		0	2	4	8	16	32	0	2	4	8	16	32	0	2	4	8			16	32
Group a. 8 wks old, kept in animal room 3 weeks before blood samples taken	91-2-4	3	3	3	1			0	0	0	0			0	0	0	0			40	0
	"	3	3	3	1			0	0	0	0			0	0	0	0			40	0
	96-7-8	2	3	1	±	0	0							1	1	0	0	0	0	20	±
	"	2	4	4	±	0	0							0	0	0	0	0	0	40	20
Group b. 5 wks old, bled when received from dealer	"	2	3	2	0	0	0							2	3	±	0	0	0	40	20
	"	0	1	1	0	0	0							0	0	0	0	0	0	+	0
	"	0	2	4	4	±	0							0	4	4	3	0	0	80	80
	"	3	4	4	3	0	0							3	4	4	±	0	0	80	>40
	"	4	4	4	3	0	0							3	4	3	0	0	0	80	>40
	"	4	4	3	2	0	0							1	1	0	0	0	0	40	+
	"	±	±	±	0	0	0							0	0	0	0	0	0	0	0
	"	2	4	4	3	0	0							1	3	4	2	0	0	80	80
	"	4	4	4	3	±	0							3	4	3	0	0	0	80	>40
	"	2	4	4	3	±	0							0	1	2	±	0	0	80	40
Group c. Pooled sera from 3 rats 5 wks old	100	0	0	0	0	0														0	0

* "Normal" antigen = preparation from normal brain and spinal cord. Virus preparations 96-7-8 and 100 were prepared as previously described and were used at a concentration of 10-5 g nitrogen per ml.

TABLE IV.

Complement-fixation Tests on Human Convalescent Sera with Concentrated Lansing Virus and Normal Cotton Rat Tissue Antigen.

Name	Case history*	Date of blood sample	Antigen†	Serum dilution, 1:							Serum Control	Titer
				0	2	4	8	16	32	64		
M.K.	♀, 23 yr, A.P. 8-1-46	1-28	Virus				4	4	1	0	0	>160
		"	"		4	4	4	4	2		0	320
		"	Normal		0	0	0	0	0		0	0
		3-5	Virus	4	4	4	4	4	3		0	320
		"	Normal	0	0	±	0	0	0		0	0
J.I.	♀, 10 mo., A.P. 7-20-46	1-28	Virus	2	4	4	4	2	0		0	160
		"	Normal	0	0	0	0	0	0		0	0
		3-5	Virus	1	3	3	1	0	0		0	40
		"	Normal	0	0	0	0	0	0		0	0
J.M.	♂, 28 yr, A.P. 10-11-46	1-28	Virus	2	4	4	2	±	0		0	80
		"	Normal	0	0	0	0	0	0		0	0
		3-5	Virus	1	2	2	1	0	0		0	40
		"	Normal	0	0	0	0	0	0		0	0
R.He.	♂, 33 yr, A.P. 9-7-46	1-28	Virus	1	1	1	1	1	±		0	+
R.H.	♂, A.P. 8-46	"	"	1	1	1	1	±	±		0	+
S.McI.	♀, 4 yr, A.P. 8-13-46	"	"	±	±	±	±	±	±		0	0
N.K.	♀, 28 yr, A.P. 8-16-46	"	"	0	0	0	0	0	0		0	0
F.O'C.	20 yr, A.P. 9-16-46	"	"	0	0	0	0	0	0		0	0
N.W.	♂, 21 yr, A.P. 6-5-46	"	"	0	0	0	0	0	0		0	0
D.W.	♀, 4 yr, bulbo-spinal poliomyelitis, 10-1-46	"	"	0	0	0	0	0	0		0	0
F.C.	♂, 10 yr, A.P. 7-15-46	"	"	0	0	0	0	0	0		0	0
G.J.	♂, 26 yr, A.P. 7-15-46	"	"	±	±	±	±	±	±		0	0
G.H.	♂, 6 yr, A.P. 9-1-46	"	" 100	1	2	3	2	0			0	80
M.P.	♂, 7 yr acute poliomyelitis 2-4-47, expired 2-5-47	2-4	"	0	0	0	0	0	0		0	0
			96-7-8									
			Normal	0	0	0	0	0	0		0	0

* A.P. = Anterior poliomyelitis.

† The virus preparation used, with the exceptions stated, was 91-2-4; the normal cotton rat antigen was the same as that described in the text.

tion had been demonstrated between virus and its antibody. However, unequivocal proof was provided when sera from a Lansing convalescent rhesus monkey and from 13 convalescent human cases† were examined for complement-fixing antibodies with concentrated Lansing virus and with the normal brain and spinal cord concentrate from cotton rats.

Blood was obtained from the monkey 3 weeks after it developed a partial paralysis following intracerebral inoculation with Lansing virus. The serum fixed complement when concentrated virus was used as antigen, giving a titer of 320. No fixation occurred when the concentrate from normal rat tissue was used as antigen, and sera from 6 normal

monkeys were negative against both the virus and the antigen from normal rat tissues.

The results with 13 recent human convalescent sera and with that from a fatal case are recorded in Table IV. In those cases where positive complement-fixation was found, the tests were repeated several times with essentially identical results. Six of the 14 sera were positive, giving titers that ranged up to 320, seven were negative as was also that from the fatal case obtained the same day final diagnosis was made. In the positive tests there was no question about complement-fixation with a normal brain protein impurity in the concentrated virus since negative results were found when the preparation from normal rat tissues was used as antigen.

Second samples of blood from three subjects giving high titers were reexamined about 5 weeks after the first samples had been tested. As shown in Table IV, these again gave pos-

† The samples of monkey blood were kindly provided through the cooperation of Professor E. W. Schultz and Dr. S. C. White and the human convalescent sera by Drs. H. K. Faber, L. A. Luz, and R. D. Cutter.

TABLE V.
Comparison of Neutralization and Complement-fixation Tests with Human Sera.

Serum		Dilution used	I.D. 50*	Neutralization titer†	Complement-fixation titer
M.K.	Convalescent	1:1000	10-7.9	10-4.9	320
R.He.	"	1:100	10-9.0	10-7.0	+
D.W.	"	"	10-8.8	10-6.8	0
M.P.	Fatal case	"	10-10.3	10-8.3	0

* 1 I.D. 50 = g of virus nitrogen required in a dose of 0.05 ml to infect 50% of animals when the dilution of serum given was used.

† Neutralization titer is defined as grams of virus nitrogen neutralized to the 50% end point by 1 ml of undiluted serum.

itive tests with titers that were comparable to those obtained the first time.

A comparison of the histories of the patients failed to show any correlation of positive or negative reactions with either age of the patient or length of time between onset of the disease and the testing of blood samples. The demonstration that approximately 45% of the group of patients possessed humoral antibodies that react with Lansing virus emphasizes the importance of this or a closely related strain as one responsible for poliomyelitis in humans. In the case of the negative reactions it is possible either that the individuals in question failed to produce humoral antibodies in sufficient concentration to be detected or that they may have been infected by a poliomyelitis virus immunologically unrelated to the Lansing strain.⁹

As shown in Table IV the samples of convalescent human sera showed a wide range in their complement-fixing titers. It was of interest to determine if complement-fixation titer could be correlated with ability to neutralize active virus. Neutralization tests were accordingly made on four human sera, namely the one giving the strongest reaction (M.K.), one showing weak complement-fixation (R. He.), and 2 giving no reaction (D. W. and M. P.). The tests were set up using a 1:100 or a 1:1000 dilution of serum in saline with serial dilutions of virus in steps of ten from 10^{-11} g nitrogen per ml to 10^{-5} g nitrogen per ml. The mixtures were incubated at 37° for one-half hour, and 0.05 ml of each dilution was injected intracerebrally

into each of 5 young cotton rats. The infectivity of the mixtures was determined by the incidence of poliomyelitis and the amount of active virus necessary to infect 50% of the animals calculated according to the method of Reed and Muench.¹⁰ The results which are recorded in Table V showed that over a hundred times as much virus was neutralized by serum M. K. (complement-fixation titer 320) as by either the weakly positive or the negative convalescent sera. Of interest was the fact that the sample from the fatal case, M. P., neutralized less than one-tenth as much virus as the negative convalescent serum. These results are, therefore, in agreement, with the view that the same antibody was involved in complement-fixation and in virus neutralization.

Conclusions. The experiments presented above show that complement-fixing antibodies can be demonstrated in the blood of rats immunized with formalized Lansing virus, in convalescent monkey serum, and in some convalescent human sera when concentrated Lansing virus from cotton rats is used as antigen. The results with rat sera are complicated by the presence of antibodies against components of normal brain and spinal cord in both immune and normal animals, but the evidence indicates that the primary reaction with immune sera is between antibody and virus. Immunization as a result of injection of formalized virus is, therefore, associated with the production of serologically detectable antibodies to active virus. The experiments with the convalescent monkey and human

⁹ Sabin, A. B., *J. Am. Med. Assn.*, 1947, **134**, 749.

¹⁰ Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

sera provide definite proof that positive complement fixation can be demonstrated when concentrated Lansing virus prepared as described² is used as the antigen. These latter

results further support the importance of the Lansing virus or an antigenically related strain as one responsible for the human disease.

16101

Influence of Pyribenzamine and Antistine upon the Action of Hyaluronidase.

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It is the general belief that the antianaphylactic and anti-allergic properties of Pyribenzamine and similar substances are due primarily to their ability to nullify the pathologic effects of histamine. This belief is based upon the following premises: First, that the symptoms of anaphylaxis and certain allergic diseases are produced by histamine, which is released during the antigen-antibody reaction; and secondly, that the most outstanding property of Pyribenzamine and related substances is their antihistaminic power (Mayer, Huttner and Scholz,¹ Mayer²).

Many clinical observations on the activity of Pyribenzamine and similar substances in various forms of allergic manifestations appear to sustain this explanation. The effectiveness of antihistaminic therapy depends in a large measure upon the type of allergy against which it is used, and antihistaminics are most effective in conditions in which histamine release is suggested by experimental findings, namely in anaphylaxis, serum disease, urticaria or hay fever. On the other hand, if the activity of these substances is due solely to the inhibition of histamine, then they should be ineffective in allergic dermatitis or eczema, since none of the clinical symptoms of contact dermatitis or eczema appear to have any resemblance to the known histamine symptoms. To date, all attempts to produce these forms of skin reactions by the administration

of histamine have been unsuccessful.

But in spite of these facts, there are numerous clinical reports citing the effectiveness of the various antihistaminics in contact dermatitis and atopic eczema (Feinberg,³ Chobot⁴), and one of us has shown that Pyribenzamine exerts a considerable prophylactic activity in experimental contact dermatitis of guinea pigs (Mayer^{5,6}). Since the effectiveness in these epidermal sensitizations is difficult to explain on the basis of inhibition of histamine, it appears more likely that antihistaminic substances act in these cases by virtue of a pharmacodynamic property different from their action upon proven histamine effects. All antihistaminic substances developed thus far possess, in addition to their antihistaminic power, various degrees of antispasmodic and local anesthetic activities. It is possible that the curative effect in dermatitis and eczema may be explained as being due to the local anesthetic property, although several observations contradict this view.

Recent investigations on the mechanism of epidermal inflammations due to the action of chemical or physical influences have pointed to the importance of certain amines, other than histamine, and of substances such as leukotaxine, pyrexin and necrosin (Menkin⁷). Although the significance of these substances

¹ Mayer, R. L., Huttner, G. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

² Mayer, R. L., *J. Allergy*, 1946, **17**, 153.

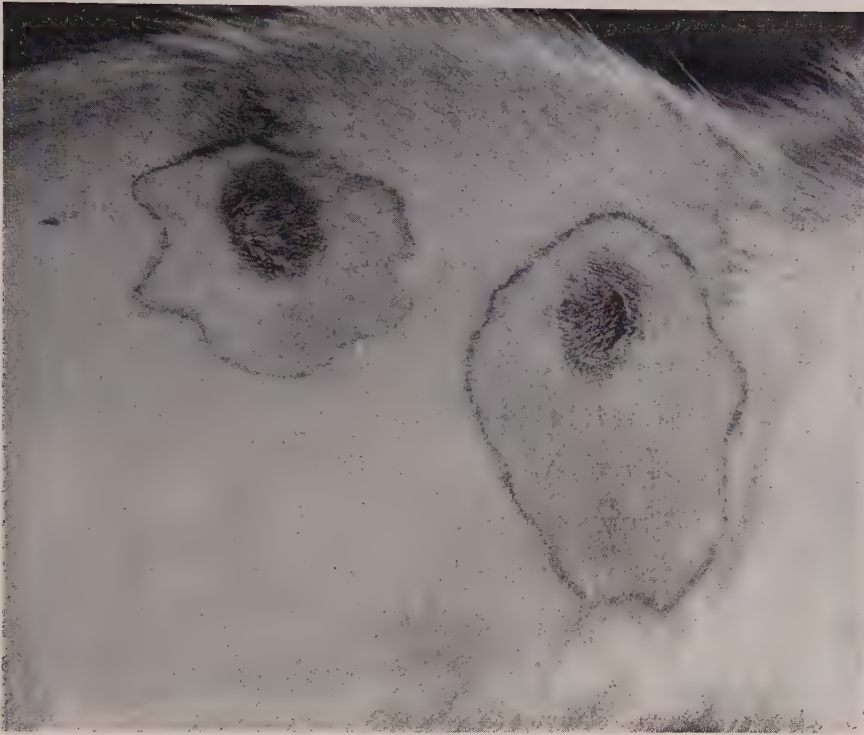
³ Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 702.

⁴ Chobot, R., *J. Allergy*, 1947, **18**, 76.

⁵ Mayer, R. L., *J. Invest. Dermat.*, 1947, **8**, 67.

⁶ Mayer, R. L., *Ann. Allergy*, 1947, **5**, 113.

⁷ Menkin, V., *Arch. Path.*, 1946, **41**, 376.



Tail

Head

FIG. 1.

Rat No. 32, right side, before Pyribenzamine. Tail: India ink plus saline. Head: India ink plus 1% hyaluronidase.

Note: Dark spot upper center is due to the subsequent injection of Pyribenzamine.

is somewhat uncertain, Cullumbine⁸ has investigated the influence of antihistaminic substances on the action of leukotaxine; he could not detect any anti-inflammatory activity.

On the other hand, various inflammatory processes produced by strongly invasive micro-organisms have been associated with the release, from these organisms, of a specific enzyme, namely hyaluronidase (Duran-Reynals⁹). So far, very little is known concerning the role which this enzyme plays in non-bacterial inflammations of the skin. Since relatively large concentrations of hyaluronidase occur in the normal skin, this may indicate a more general function. (Meyer *et al.*,¹⁰ Meyer¹¹) Certain of the observations described below indicate that hyaluronidase may be associated with certain phases of skin inflammations, especially of allergic non-bacterial nature.

We have directed our investigations in this direction and have studied the influence of Pyribenzamine and antistine upon the activity of hyaluronidase both as a spreading factor and as a possible factor responsible for allergic inflammation of the skin.

Experimental. A. Spreading of India ink in rats. The effect of Pyribenzamine and antistine upon the diffusion of India ink in the skin of albino rats in the presence and absence of hyaluronidase was investigated according to the method described by Duran-Reynals⁸ and Cahen and Granier.¹² One hundred and twenty male and female rats, each

⁸ Cullumbine, H., *Nature*, 1947, **159**, 841.

⁹ Duran-Reynals, F., *Bact. Rev.*, 1946, **6**, 197.

¹⁰ Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.

¹¹ Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

¹² Cahen, R. L., and Granier, M., *Yale J. Biol. and Med.*, 1944, **16**, 257.



Tail

Head*

FIG. 2.

Rat No. 32, left side, after 37.5 mg of Pyribenzamine. Tail: India ink plus saline. Head: India ink plus 1% hyaluronidase.

* This photograph has purposely been reversed in order to permit a comparison of Fig. 1 and 2.

weighing between 120 and 220 g, were treated simultaneously at 2 sites on the right flank; one site being injected intradermally with a mixture of 0.05 cc of Higgins India ink and 0.10 cc physiological saline, and the other site with a mixture of 0.05 cc of ink and 0.10 cc 1% hyaluronidase solution. The hyaluronidase preparation* used in these experiments contained 150 turbidity-reducing units per milligram. Twenty hours later the areas were outlined in ink, traced on cellophane sheets, and measured with a planimeter.

Having thus determined the *in vivo* activity of the hyaluronidase preparation, the animals were subcutaneously injected on the right side 24 hours after the India ink injections with a solution of Pyribenzamine or antistine in doses of 15, 37.5 and 75 mg per kg of body

weight respectively; the highest dose representing $\frac{1}{3}$ to $\frac{1}{5}$ of the LD_{50} for rats (Mayer *et al.*¹³).

Twenty minutes after the administration of the antihistaminic, the intradermal injections of India ink plus saline, and India ink plus hyaluronidase were repeated on the corresponding areas of the left flank. The areas of spreading were measured as outlined above.

The results of this India ink test, given in Table I, show that the antihistaminic substances Pyribenzamine and antistine strongly reduced both the extent of the normal spreading of injected India ink, as well as the spread produced in the presence of hyaluronidase. Doses of 15, 37.5 and 75 mg/kg of Pyribenzamine restricted the area of spreading pro-

* We are indebted to Dr. E. Schwenk, of the Schering Corp., who kindly supplied the material.

¹³ Mayer, R. L., Hays, H. W., Brousseau, D., Mathieson, D., Rennick, B., and Yonkman, F. F., *J. Lab. Clin. Med.*, 1946, **31**, 749.

duced by hyaluronidase by 6%, 21% and 44% respectively over that of the control areas. Similar doses of antistine restricted the spreading by 6%, 20% and 51% respectively. Fig. 2 shows the decrease of the hyaluronidase spreading effect after injection of 37.5 mg/kg Pyribenzamine, in contrast to the hyaluronidase effect on the same animal before Pyribenzamine treatment, as shown in Fig. 1.

Morphine sulfate when tested according to the method of Cahen and Granier¹² in doses of 25 and 32 mg/kg, restricted the increase of spreading by 46% and 47% respectively.

B. Effect of Antihistaminics Upon the Action of Hyaluronidase in Experimental Sensitizations. 1. Action of Hyaluronidase upon Allergic Inflammation. We sensitized 18 guinea pigs to paraphenylenediamine by treatment with 10% paraphenylenediamine ointment according to a method previously described (Mayer¹⁴) and challenged them 3 weeks later with an intradermal injection of 0.10 ml of 0.75% paraphenylenediamine solution.

The paraphenylenediamine used for the challenge was freshly recrystallized and solutions prepared in the absence of oxygen, in order to minimize the formation of oxidation products which could destroy the hyaluronidase. Since the solutions were slightly tinted in spite of these precautions, 10% horse serum was added immediately before the injections as a further precaution, the serum proteins combining with and simultaneously reducing the small amount of oxidation product present. Although it is known that certain sera inhibit the action of hyaluronidase (McClean¹⁵), Hechter and Scully¹⁶ have recently shown that serum interferes only after incubation; in our experiments no significant diminution of the spreading effect took place.

The challenge injections with antigen alone and with antigen-hyaluronidase mixtures were performed in the same manner as described above for the India ink experiments in rats. The areas of inflammation and infiltration,

TABLE I.
Effect of Pyribenzamine and Antistine upon the Spreading of India Ink in the Presence and Absence of Hyaluronidase.
20 rats tested in each experiment.

Exp.	Dosage (mg/kg)	Reduction of spreading area	
		India ink alone	India ink plus hyaluronidase
	Pyribenzamine	%	%
1	15	14.05 $\pm$ 4.21*	6.25 $\pm$ 6.39
2	37.5	18.70 $\pm$ 5.20	21.10 $\pm$ 3.77
3	75	47.85 $\pm$ 2.53	43.95 $\pm$ 3.73
	Antistine		
4	15	13.65 $\pm$ 5.73	11.10 $\pm$ 4.77
5	37.5	31.75 $\pm$ 5.22	20.00 $\pm$ 4.23
6	75	28.10 $\pm$ 4.99	37.00 $\pm$ 3.22

* $\pm$ values represent probable error of the mean.

varying from 6 mm to 25 mm in diameter, were measured and at the same time, the intensity of infiltration and redness noted by symbols as indicated in Table II.

The results obtained indicate that hyaluronidase increases the spread of the allergic epidermal reaction in sensitized guinea pigs, as it increases the spread of India ink in rats. The average challenge reaction in sensitized animals measured 2.3 mm² when the antigen was injected alone, and 16.9 mm² when it was combined with hyaluronidase. Simultaneously, there was a marked increase in the intensity of the allergic inflammation which was even more significant than the increase in the size of the reaction. The intensity increased from a + reaction to a +++ or ++++ reaction in the presence of hyaluronidase, in spite of the fact that the greater spread, as proven by the increased area of inflammation, had resulted in a marked dilution of the antigen concentration at the site of reaction.

2. Influence of Pyribenzamine Upon the Hyaluronidase Effect in Allergic Inflammations. a. Sensitization to paraphenylenediamine. Twenty-four hours after the spreading test was performed these animals received 15 mg/kg Pyribenzamine subcutaneously on the same flank. Twenty minutes later they were retested on the opposite flank with paraphenylenediamine and a paraphenylenediamine-hyaluronidase horse serum mixture in the same manner. Since the challenge reaction is in this case an epidermal inflammation of the contact dermatitis or tuberculin type, which

¹⁴ Mayer, R. L., *Arch. f. Dermatol. u. Syph.*, 1931, **163**, 223.

¹⁵ McClean, D., *J. Path. and Bact.*, 1942, **54**, 284.

¹⁶ Hechter, O., and Scully, E. L., *J. Exp. Med.*, 1947, **86**, 19.

TABLE II.
Influence of Hyaluronidase on the Challenge Reaction in Experimental Epidermal Sensitizations to Paraphenylenediamine (PP) in Guinea Pigs.

Average size and intensity of challenge reaction					
	No. of animals	Without hyaluronidase		With hyaluronidase	
		Size in mm ²	Intensity of inflammation	Size in mm ²	Intensity of inflammation
Controls	4	0.317 $\pm$ 0.124	(+)	1.60 $\pm$ 0.14	+
Sensitized animals	18	2.3 $\pm$ 0.74	+	16.9 $\pm$ 0.52	+++ to +++++

Signs: (+) to +++++ represent varying degrees of inflammation as measured by the intensity of redness and degree of infiltration.

TABLE III.
Influence of Pyribenzamine upon the Allergic Reaction in Guinea Pigs to Paraphenylenediamine in Presence and Absence of Hyaluronidase.

Average size and intensity of local challenge reaction					
	No. of animals	Without hyaluronidase		With hyaluronidase	
		Size in mm ²	Intensity of inflammation	Size in mm ²	Intensity of inflammation
Controls	4	0	0	0.75 $\pm$ 0.48	0
Sensitized animals	18	0.27 $\pm$ 0.14	0	2.7 $\pm$ 0.54	(+)

15 mg per kg body weight of Pyribenzamine was given twice subcutaneously, namely, 15 to 20 minutes before and 4½ hours after the challenge injection of the antigen.

develops gradually, reaching a maximum only after 12 to 16 hours, an additional dose of Pyribenzamine (15 mg/kg) was injected 4½ hours later.

The results of this experiment are given in Table III. The area of the challenge reaction to the antigen alone this time was found to be to an average size of 0.27 mm², against 2.3 mm² before the Pyribenzamine injection. This reaction became even smaller than the unspecific, non-allergic reaction to the paraphenylenediamine injection in non-sensitized control animals. The average size of the challenge reaction produced in the presence of hyaluronidase was reduced from 16.9 mm² to 2.7 mm², a value close to that observed in the animals having received antigen alone (Table III). Thus the pretreatment with Pyribenzamine almost completely suppressed the allergic reaction regardless of whether the antigen was injected with or without hyaluronidase.

b. Sensitization to 2,4-dinitrochlorobenzene. In a previous study it was shown that antihistaminic substances prevent or diminish the epidermal manifestations of experimental sensitizations of guinea pigs to 2,4-dinitrochloro-

benzene (Mayer¹⁴). This type of sensitization differs from that produced by paraphenylenediamine in the clinical aspect of the challenge reaction, as well as in the histological changes produced. It was therefore of interest to investigate whether the challenge reaction in sensitizations to this nitro compound was also reinforced by hyaluronidase, as was the case in sensitizations to the diamine.

Ten guinea pigs were sensitized to 2,4-dinitrochlorobenzene by intradermal injection of 0.02 mg of antigen, and after 27 days all animals had become strongly allergic to the substance. They were then challenged intradermally with 0.1 ml containing 0.02 mg of antigen alone and with a mixture of 0.02 mg of antigen and 0.5 mg of hyaluronidase in a similar manner, as described for paraphenylenediamine. However, in this case no horse serum was added to the antigen-hyaluronidase mixture, since, according to Landsteiner¹⁷ 2,4-dinitrochlorobenzene does not react with protein at room temperature.

In this case, however, hyaluronidase neither increased the size nor the intensity of the

¹⁷ Landsteiner, K., *The Specificity of Serological Reactions*, Cambridge, Mass., 1945.

challenge reaction. It is not known yet whether this negative result is due to a destruction of hyaluronidase, or to a blocking of its action by the nitro compound, or whether this type of epidermal sensitization does not intrinsically respond to hyaluronidase.

C. Role of the Local Anesthetic Activity of Antihistaminics. Pyribenzamine and the other antihistaminic substances of similar chemical constitution have local anesthetic activity equal to or superior to that of procaine. It was therefore of interest to determine whether a local anesthetic substance with low antihistaminic activity, such as procaine, influenced the spreading effect of hyaluronidase in the same way as Pyribenzamine.

Twenty rats were tested as outlined above and the influence of procaine upon the spread of India ink investigated at two different levels, namely 75 mg/procaine per kg of body weight, which corresponded to the highest doses of Pyribenzamine used in the earlier experiments and 225 mg per kg of body weight, representing a 3-fold increase in the local anesthetic action.

Seventy-five mg/kg of procaine had no significant influence upon the spread of India ink either in the absence or presence of hyaluronidase. With 225 mg per kg procaine, the spread of India ink was decreased by 11% in the absence of hyaluronidase, and 18% in experiments performed in the presence of hyaluronidase. The activity of this high concentration of procaine could be due to an unspecific cell injury, since it was highly irritating locally and produced deep necrosis.

According to these results, procaine has a slight influence upon the normal spread of India ink, as well as on the spreading effect of hyaluronidase. It is, however, much less active than Pyribenzamine or antistine in this respect.

Discussion. The present experimentation was performed to determine (a) whether hyaluronidase plays a role in allergic, non-bacterial inflammation of the skin and (b) whether antihistaminic substances have an anti-hyaluronidase activity.

The results indicate that hyaluronidase markedly influences the epidermal challenge

reaction in guinea pigs sensitized to paraphenylenediamine, its presence increasing the size and intensity of inflammation. Furthermore, the fact that antihistaminic substances markedly decrease the effect of hyaluronidase upon the spread of India ink and upon the allergic reaction to paraphenylenediamine constitutes, we believe, reasonable indication that hyaluronidase does play a role in certain non-bacterial, especially allergic inflammation, as it does in certain bacterial processes. The direct proof for this theory, however, is still lacking since it has not been shown that hyaluronidase is liberated during the allergic inflammation of the skin (and other organs) and that, if its amount is increased during this inflammation, it is etiologically related with some phase of the allergic reaction. So far, certain technical difficulties have been encountered in the interpretation of experiments performed to elucidate this point.

The present investigation has furthermore disclosed that antihistaminic substances such as Pyribenzamine and antistine possess, in addition to their antihistaminic activity, other properties which may permit action in various manifestations thus far not associated with histamine-release. The strong anti-hyaluronidase effect, which results in a reduction of the size as well as the intensity of an allergic inflammation, may explain the activity of Pyribenzamine and similar substances in epidermal sensitizations such as eczema or contact dermatitis. It might also explain the effect antihistaminics seem to exert in certain rheumatic manifestations, where, according to Guerra, salicylates act by a similar mechanism.

It is not yet known whether this anti-hyaluronidase effect is a specific pharmacologic property of Pyribenzamine and antistine, comparable, for instance, to their specific antihistaminic power. It is quite possible that the effect is the result of a nonspecific process. On the other hand, the local anesthetic power may, to a certain extent, be associated with this activity—a question which could be decided by the use of antihistaminic substances devoid of local anesthetic power.

Conclusions. Experiments are presented which show that (a) hyaluronidase increases the intensity of allergic skin inflammation of

TABLE IV.
Influence of Procaine upon the Spreading of India Ink in the Presence and Absence of Hyaluronidase. 10 rats tested in each experiment.

Exp.	Dosage (mg/kg)	Average size of spreading		Reduction of spreading area	
		Without hyaluronidase	With hyaluronidase	Without hyaluronidase	With hyaluronidase
				%	%
1	0	5.92	8.9		
	75	5.8	8.4	3.8 ± 6.4	5.3 ± 4.5
2	0	6.1	8.8		
	225	5.3	7.0	11.1 ± 3.4	18.1 ± 3.3

the epidermal type and (b) that antihistaminic substances such as Pyribenzamine and antistine counteract the spreading effect of India ink and hyaluronidase, as well as the

latter's effect upon the allergic skin inflammation. The significance of these findings for the activity of antihistaminics in epidermal sensitizations is discussed.

16102

Isolation and Properties of Snake Erythrocyte Nuclei.

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The separation of nuclei from avian erythrocytes has been attempted by Warburg and by Miyake using the freezing and thawing technique.^{1,2} However, the nuclei obtained by this method are not well separated and usually agglutinate in a mass. Laskowski was able to prepare a stable suspension of free nuclei of chicken blood using lysolecithin in neutral phosphate buffer solution for the hemolysis with subsequent washing of the nuclei with isotonic saline.³ The poison glands of bees were ground with the lecithin emulsion which was then incubated for 24 hours at 37°C and finally filtered through a Berkefeld filter. The lysolecithin so prepared was added to the blood and after the hemolysis the nuclei were separated by centrifugation. This technique yields good preparations but the lysolecithin is not easily obtained under

standard conditions. The procurement of the bee poison and the time required are disadvantageous conditions when large amounts of blood are used for chemical analysis.

The well known hemolytic action of tyrothricin, an antibiotic obtained from *Bacillus brevis* and commercially available,* has been used in our experiments with very satisfactory results. Snake erythrocytes after rapid hemolysis liberate the nuclei which are then washed several times in 0.9% saline and 5% citric acid solution. Microscopic examination of the nuclei suspension shows that they are not agglutinated and are almost free from the cytoplasm (Fig. 1). They are easily stained with methylene blue and Giemsa. The nuclei in suspension appear microscopically smaller and more compact than in the intact erythrocytes. This observation was also reported by Laskowski for chicken blood preparations.

¹ Warburg, O., *Z. physiol. Chemie*, 1910, **70**, 413.

² Miyake, M., *Keijo J. Med.*, 1933, **4**, 247.

³ Laskowski, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **49**, 354.

* Tyrothricin 2% solution, Parke, Davis & Co.

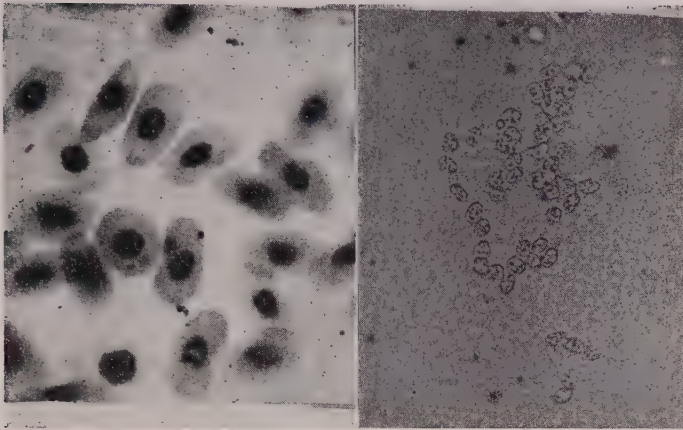


FIG. 1.

Left: Nuclei of the erythrocytes of the snake *Eudryas bifossatus* obtained with the tyrothricin hemolytic technique. Right: Blood of the same snake stained with Giemsa. $\times 810$.

Fig. 1 shows a microphotograph of the nuclei of erythrocytes of snake blood suspended in 5% citric acid in comparison with the nuclei in the intact erythrocytes stained with Giemsa.

Method. The blood of 2 poisonous and 4 non-poisonous snakes was studied (Table I). It was collected from decapitated snakes directly into large test tubes containing 0.3% potassium oxalate. The amount obtainable (usually 25 to 40 ml) depended upon the size of the reptile. After centrifugation the plasma and leucocytes were pipetted off and discarded. The erythrocytes were washed several times (5 to 7) with 0.9% NaCl solution, until the washing was water clear. The densely packed cell layer was mixed with saline in a stoppered flask to obtain a 5% suspension. To 40 ml of the erythrocyte suspension 1 ml of the tyrothricin solution (20 mg/ml)[†] was added. The suspension was mixed well immediately by inversion of the flask. After 10 minutes the hemolyzed blood was centrifuged and the whitish mass of nuclei washed twice with saline, once with 5% citric acid, and finally suspended in the

citric acid solution. For chemical analysis the citric acid treatment was omitted. The suspension of the nuclei was dehydrated with 95% alcohol in a centrifuge tube and the alcohol was evaporated under low pressure until a fine white powder was obtained. The powder was left over night in a vacuum desiccator and stored in the refrigerator. By this technique 74 mg could be prepared from 5 ml of washed snake erythrocytes. The same technique was applied successfully to the blood of fowls.

Results. Applying the saponin method of Dounce and Lan⁴ to the erythrocytes of some snakes we obtained good preparations, but the nuclei still showed a layer of stroma. The authors cited demonstrated that the nuclei from chicken erythrocytes, which are somewhat yellow in color, contain a xanthophyll pigment and probably a flavin. Since the plasma of some snakes (*Bothrops*, *Eudryas*) is very rich in riboflavin, as formerly reported by us⁵ it would be of interest to know if the nuclei of the erythrocytes also contain flavins. Using both tyrothricin and saponin techniques, we were unable to detect any flavin or carotenoid pigment in any sample exam-

[†] We wish to express our best thanks to Dr. Lauro Trabassos, Division of Zoology, Instituto Oswaldo Cruz, and Dr. Ananias Porto, Instituto Butantan, S. Paulo, for the samples of snake bloods used in this study.

⁴ Dounce, A. L., and Lan, T. H., *Science*, 1943, **97**, 584.

⁵ Villela, G. G., and Prado, J. L., *J. Biol. Chem.*, 1945, **157**, 693.

TABLE I.
Desoxyribonucleic Acid Content of Nuclei of the Snake Erythrocytes.

Common names	Scientific names	No. of samples	Mg of desoxyribonucleic acid in 100 mg dry nuclei
Non poisonous snakes:			
Limpa-campo	<i>Eudryas bifossatus</i>	3	64
Giboia	<i>Boa</i> sp.	1	62
Caninana	<i>Spillotis pullatus</i>	2	31
Boipeva	<i>Xenodon merrimii</i>	1	44
Poisonous snakes:			
Rattlesnake (Cascavel)	<i>Crotalus terrificus</i>	3	59
Jararaca	<i>Brothrops jararaca</i>	4	56

ined. The ether extract and the remaining nuclei mass showed only a slight white fluorescence. Thiamin and nicotinamide assays on the dry nuclei showed values averaging 80 and 122 γ /g respectively (4 samples analyzed). The microbiological methods of Sarett and Cheldelin⁶ for thiamin and of Krehl, Strong and Elvehjem⁷ for nicotinamide were employed. The nuclei assayed were only saline-washed. Citric acid-treated nuclei are not suitable for analysis by these methods.

The desoxyribonucleic acid content of the nuclei was determined by use of the Dische reaction, as adapted by Dounce to permit spectrophotometric measurement of the blue color.⁸ In a test tube 2 mg of the alcohol-dried nuclei were suspended in 0.5 ml of distilled water and 1.0 ml of the Dische reagent. The tubes were immersed in a boiling water bath and 8.5 ml of the blank reagent (sulfuric-acetic acid mixture) was added. The slight turbidity appearing 1 to 2 minutes later was easily removed by the addition of 0.1 to 0.2 g of celite (Hyflo-supercel) and subsequent filtration or centrifugation. The clear blue color was measured in a Lumetron photocolormeter using a 530 $m\mu$ filter. The blank value was subtracted from the unknown. To the tube containing the blank

were added 1.0 ml of the Dische reagent and 7.5 ml of the blank reagent (sulfuric-acetic mixture) so that the total volume in the blank and unknown tubes was 10 ml. The color was stable for only 20 minutes and, therefore, all the operations had to be run in a short time. The values, referred to as desoxyribonucleic acid, were calculated from a calibrated curve made with pure sodium desoxyribonucleate prepared from normal liver nuclei according to a modified Hammarsten method and tested both spectrophotometrically and chemically (N : P ratio = 2.34%). The averages obtained for the nuclei of the different snake erythrocytes are shown in Table I.†

The percentage of desoxyribonucleic acid found in the nuclei of snakes showed higher values than those reported by Dounce for the nuclei of chicken erythrocytes and of rat liver (about 45%).^{4,8} The total lipid content was determined only for the nuclei of the snakes *Spillotis pullatus* and *Eudryas bifossatus* and averaged 12.7%. Dounce and Lan reported for the chicken the total lipid content of about 14%.⁴

Summary. A new technique for the isolation of the snake erythrocyte nuclei using tyrothricin as an hemolytic agent is described. Some properties and chemical data are reported. The desoxyribonucleic acid content of the erythrocyte nuclei for the 6 kinds of snakes studied was higher than that reported for chicken erythrocytes and liver rat nuclei.

⁶ Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, 1944, **155**, 153.

⁷ Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 471.

⁸ Dounce, A. L., *J. Biol. Chem.*, 1943, **151**, 231.

Attempted Infection of the Hen and Man with the Sporozoites of *Plasmodium cathemerium* 3H2.*

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Huff and Coulston¹ reported their inability to find cryptozoites in canaries inoculated with the sporozoites of *Plasmodium gallinaceum*, a parasite to which the chick is quite susceptible, and also their failure to establish infections in canaries by the inoculation of blood from chickens infected with this organism. The present communication deals with a reversed experiment, *i.e.*, the attempt to infect the chicken with both the sporozoites and the trophozoites of a parasite that is highly virulent in canaries. The organism used in these trials was the *P. cathemerium* 3H2 that has been continuously maintained through mosquito-canary-mosquito passages in this laboratory since February, 1937. In addition there will be placed on record here an attempt to infect man with this organism, which I believe has not previously been reported.

First Trial. Using heparin as anticoagulant, 5 cc of blood was drawn by cardiac puncture from each of 3 full-grown white Leghorn hens and the plasma obtained after pooling the samples. Then 100 *Culex pipiens* mosquitoes that had fed two weeks previously on canaries whose blood contained numerous gametocytes of *P. cathemerium* 3H2 were stunned in a flask, ground in 2.5 cc of the plasma, and washed through 3 layers of gauze with another 2.0 cc of plasma; the volume of the filtrate was then made up to 5 cc with additional plasma. Of this filtrate, 0.1 cc (representing the sporozoites from 2 mosquitoes) was immediately injected intramuscularly into each of 4 clean canaries and 0.3

cc (6 mosquitoes) intramuscularly into each of 4 white Leghorn hens. Plasmodia were found in the peripheral blood of the canaries on the 7th, 7th, 8th and 9th days, respectively, but the hens remained negative through 17 days. On the 18th day the blood drawn by cardiac puncture from each of the 4 hens was pooled and 0.1 cc injected intramuscularly into each of 4 clean canaries; the blood of these birds remained negative through 16 days but all 4 birds subsequently became infected when challenged by the bites of infected mosquitoes.

Second Trial. Hen blood was obtained as in the preceding trial but it was allowed to stand after cooling at room temperature for one hour before centrifuging to obtain the plasma. Thereafter all steps were as in the preceding trial and the results were precisely the same.

Third Trial. One hundred infected mosquitoes were ground in 1.0 cc of Locke's solution for 30 seconds and the filtrate made up to 1.0 cc with Locke's solution; 0.2 cc of this filtrate was then diluted to 2.0 cc with Locke's solution and 2 hens were each injected intramuscularly with 0.5 cc (5 mosquitoes) of this mixture and 2 canaries with 0.1 cc (1 mosquito). Again the same results were obtained.

Fourth and Fifth Trials. These were repetitions of the immediately preceding trial, with the same results.

Sixth Trial. In this trial the mosquito-Locke filtrate was added to whole canary blood and rotated in the incubator at 41.5°C for 2½ hours before injecting into the hens and canaries. The results were the same as in all the preceding trials.

Seventh Trial. On May 8, 1947, D.P. and H.B. were bitten on the forearm by mosquitoes infected with *P. cathemerium* 3H2 in the

* This research was supported in part by a grant from the Therapeutic Research Committee of the Council on Pharmacy and Chemistry of the American Medical Association.

¹ Huff, C. G., and Coulston, F., *J. Infect. Dis.*, 1946, **78**, 99.

usual way on April 23; D.P. received 11 bites and H.B. 17 bites. The first peripheral blood smears were examined on May 14 and thereafter on May 16, 20, 23, 27 and 30; all the smears were negative. On June 19 (6 weeks after the infective bites), 5.0 cc of blood were drawn from each of the subjects, pooled, and 0.1 cc at once injected into each of 5 canaries. The blood of these canaries remained negative through 16 days of smearing, but it subsequently became positive after the usual incubation period when the birds were challenged by the bites of infected mosquitoes.

Summary. The sporozoites of *P. cathe-merium* 3H2, an organism causing severe infections in canaries, were injected intramuscularly in large numbers into full-grown barnyard hens. In the respective trials the injectum consisted of (a) the gauze filtrate of infected mosquitoes ground in fresh hen plasma; (b) the gauze filtrate of infected mos-

quitoes ground in hen plasma obtained after allowing the drawn blood to stand for one hour at room temperature; (c) the gauze filtrate of infected mosquitoes ground in Locke's solution; (d) whole canary blood to which was added the gauze filtrate of infected mosquitoes ground in Locke's solution, the mixture being incubated at the temperature of hen blood for 2½ hours before using for injection. None of the injected hens in any of the trials became infected and all of the canaries injected as controls did become infected.

Two human volunteers were bitten, respectively, by 11 and 17 mosquitoes infected with *P. cathe-merium* 3H2; neither of the individuals became infected so far as could be determined by a study of peripheral blood smears and subinoculation of blood into canaries.

16104

Therapeutic Efficacy in Experimental Syphilis of Eight Daily Injections of Penicillin in Beeswax-Peanut Oil.*

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Many methods have been developed to prolong the therapeutic activity of penicillin preparations, but the most effective of these appears to be the use of calcium penicillin in beeswax and peanut oil.¹ Romansky² has demonstrated that therapeutic penicillin levels are maintained in man for approximately 24 hours following the single injection of 300,000 units of calcium penicillin in 4.8% beeswax

by weight in peanut oil, contained in 1 cc. From a practical standpoint the use of this preparation permits active therapy to be carried out on an ambulatory basis. The advantages of such a procedure in the treatment of syphilis are obvious.

The present report summarizes the results of an experiment designed to assay the effectiveness of calcium penicillin in beeswax and peanut oil in the treatment of experimental rabbit syphilis, when the calculated total dose is administered in 8 daily injections.

Material and Methods. Male rabbits weighing from 2.5 to 4.2 kilograms were utilized. They were housed in individual cages and maintained on commercial pellets

* This report represents work performed under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the New Britain General Hospital.

¹ Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

² Romansky, M. J., and Rittman, G. E., *New England J. Med.*, 1945, **233**, 582.

augmented by a free supply of water. The environmental temperature was stabilized through air-conditioning at a level never exceeding 75°F. Bilateral intra-testicular inoculations were carried out with saline emulsions of active syphilitic orchitic material from routine passage animals infected with the Nichols strain of *Tr. pallidum*. The inoculated animals were then observed for the development of orchitis, and dark-field examinations were performed when clinically indicated. Only darkfield positive animals were included in the treatment groups.

Treatment was begun 6 weeks following inoculation. All animals were weighed on the day prior to the beginning of treatment and the total dosage for each animal was calculated on the basis of these weights. The total amount of drug was intramuscularly injected in 8 equally divided daily injections, through the shaved skin of the hind quarters. The preparation consisted of calcium penicillin in 4.8% beeswax (by weight) in peanut oil, prepared and kindly supplied to us by Dr. Harry Eagle. Three dilutions were utilized. The first 2 were labelled "Abbott-Eagle Lot No. 2" and contained 125 units and 1,000 units of calcium penicillin per cc respectively. The third contained 8,000 units of calcium penicillin per cc and was labelled "Squibb." The Squibb preparation was employed only in the highest dosage group, *i.e.*, 16,000 units per kilo. The Abbott material was used in all other dosages.

The animals were observed for 4 months following the completion of treatment. During this period frequent clinical examinations were conducted and all suspicious lesions were examined by darkfield. If positive, the animal was considered to be a treatment failure and discarded. At the end of 4 months all surviving animals were sacrificed, and the aseptically removed popliteal lymph nodes from each animal were emulsified with equal parts of saline and rabbit serum. The resulting emulsion prepared from each rabbit was then inoculated into each testicle of 2 normal animals. These were held for 3 months and observed by frequent clinical examinations. Suspicious lesions were dark-

fielded, and when this examination was positive, the parent animal from whom the inoculated popliteal lymph nodes were secured, was deemed to be a treatment failure. At the end of 3 months the surviving transfer animals were sacrificed and the sites of inoculation examined by darkfield for the presence of treponemes. If these were found, the parent animal was considered to be a treatment failure. If this final examination was negative, the parent animal was listed as a treatment success.

There were 7 groups of treated animals. Treatment varied between groups from a total dosage of 250 units to 16,000 units per kilo of body weight. Two different experiments were conducted. The first series of animals was inoculated on February 14, 1946, and the second on September 6, 1946. The results in these 2 series were not significantly different. They were therefore combined and are reported here as a single experiment.

Results. The final results are summarized in the accompanying table. It will be noted that all of 4 rabbits receiving a total dose of 250 units per kilo were treatment failures, while all of 14 animals receiving 8,000 or more units per kilo total dose were treatment successes. There is a fairly uniform increase in the ratio of successes with increasing dosage. The CD_{50} or the amount of drug required to cure 50% of the animals, when calculated according to the Reed-Muench³ formula, was 1,070 units per kilo.

Discussion. Employing the identical method of assay, Eagle, Magnuson and Fleischman⁴ have reported a CD_{50} of 1,600 units per kilo when sodium penicillin in aqueous solution was employed, the total dosage having been given in 5 injections per day for 4 days. Penicillin G, injected every 4 hours for 24 treatments has been found⁵ to result in a CD_{50} of 1,450 and 1,600 units per kilo.

³ Reed, L. J., and Muench, W., *Am. J. Hyg.*, 1938, **27**, 493.

⁴ Eagle, H., Magnuson, H. J., and Fleischman, R., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 168.

⁵ Arnold, R. C., Boak, R. A., Carpenter, C. M., Chesney, A. M., Fleming, W. L., Gueft, B., Mahoney, J. F., and Rosahn, P. D., *Am. J. Syph., Gon. and Ven. Dis.*, in press.

TABLE I.
Results of Treatment of Experimental Rabbit Syphilis with calcium penicillin in Beeswax and Peanut Oil. Total calculated dose given in 8 daily injections.

No. of animals	Total dosage units/kg	Failed	Cured	Total Failed*	Total Cured*	% cured*
4	250	4	0	16	0	0
7	500	5	2	12	2	14
9	1000	5	4	7	6	46
9	2000	1	8	2	14	87
9	4000	1	8	1	22	95
9	8000	0	9	0	31	100
5	16000	0	5	0	36	100

* After Reed and Muench.³

Our results with 8 daily injections of calcium penicillin in beeswax and peanut oil were essentially no different from these reported for sodium penicillin and for penicillin G.

Fleming⁶ assayed a commercial preparation of penicillin in beeswax and peanut oil in the same manner as that reported herein, but his schedule employed 16 daily injections instead of 8. The CD_{50} in his experiment was 1,400 units per kilo. This value is not significantly different from our own findings of 1,070 units per kilo, when the two results are compared by the method of Behrens⁷ (difference = 1.34 ± 1.07 , $t = 1.25$). It is apparent that

⁶ Fleming, W. L., presented at a symposium, *Recent Advances in the Investigation of Venereal Disease*, Washington, D.C., Apr. 17, 1947.

⁷ Behrens, B., quoted by Gaddum, J. H., Reports on Biological Standards III. Methods of Biological Assay Depending on a Quantal Response, *Medical Research Council, London*, Special Report Series, No. 183, 1933, p. 26.

the total curative dose (CD_{50}) is not significantly altered by increasing the number of daily injections from 8 to 16. This observation may have important clinical implications in the treatment of human syphilis.

Summary. Calcium penicillin in beeswax and peanut oil was employed to treat groups of rabbits infected with *Tr. pallidum*. The calculated total dose based on body weight was administered in 8 daily injections. A CD_{50} of 1,070 units per kilo of body weight was observed. This value does not differ significantly from a CD_{50} of 1,400 units per kilo reported by Fleming following administration of a similar preparation in 16 equal daily doses. It was concluded that when calcium penicillin in beeswax-peanut oil is employed in the treatment of rabbit syphilis, increasing the number of daily injections from 8 to 16 does not alter the total curative dose (CD_{50}).

A Search for *Treponema pallidum* in the Lymph Nodes of the Syphilitic Mouse.*

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In the course of an investigation of experimental mouse syphilis, it was found important to be certain that all the mice inoculated with syphilis actually became infected and harbored the *Treponema pallidum*. As Kolle and Schlossberger¹ first showed in 1926, symptoms do not appear despite an infection which persists throughout the life of the mouse. One method of ascertaining the presence of infection is to subinoculate mouse tissues into rabbit testicle. A darkfield positive syphiloma will usually develop after 20 to 80 days if the inoculated tissues contained viable *Treponema pallidum* in amounts sufficient to infect. This is a cumbersome method and the question was raised whether the treponemes might be easily found by darkfield search of excised node tissue, as the nodes are known to be highly virulent. Also, Jahnel and Prigge² demonstrated the presence of *Treponema pallidum* in the lymph nodes of the infected mouse by using silver impregnation methods.

Levaditi and Rousset-Chabaud³ examined skin biopsies of syphilitic mice impregnated by Stroesco's silver method, to exclude non-infected mice from therapy trials. However, this method uses fixed tissue and the *Treponema pallidum* may be confused with the *Spirillum morsus muris* or other spirochetes often found in the asymptomatic mice. Two observers have reported finding the *Treponema* in nodes by darkfield examination.

Kato⁴ was the first in 1931 and Bessemans and his co-workers Van Haelst and De Moor have repeatedly found and counted the treponemes in mouse lymph nodes^{5,6} after extremely laborious searches.

Kato reported finding the *Treponema pallidum* frequently in the iliac nodes of mice inoculated intrascrotally one to 40 days previously with rabbit chancre tissue. His method was to divide the node into 5 fragments, emulsify each fragment in a drop of normal saline, and search each fragment with the darkfield microscope, examining 50 fields per fragment, or a total of 250 fields per node. His results are given in Table I.

Van Haelst⁶ found it necessary to examine from 500 to 3,000 darkfields of node tissue emulsified in such a way as to allow enumeration of the treponemes seen. He found that almost all nodes examined, from 56 to 425 days after infection, contained visible characteristic treponemes. Karrenberg⁷ attempted to repeat Kato's findings and reported failure in a very complete review of mouse syphilis. He mentioned seeing "spirochetoid" movements but no treponemes. The apparent ease with which Kato found treponemes led to the present attempt to use his method. It was hoped that examination of an excised mouse lymph node would suffice to establish the presence of syphilitic infection in the mouse, and that rabbit inoculations of control mice

* This report represents work performed under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the New Britain General Hospital.

¹ Kolle, W., and Schlossberger, H., *Deutsche Med. Wchnschr.*, 1926, **52**, 1245.

² Jahnel, F., and Prigge, R., *Deutsche Med. Wchnschr.*, 1929, **55**, 694.

³ Levaditi, C., and Rousset-Chabaud, D., *Bull. Acad. de Méd. Paris*, 1941, **124**, 176.

⁴ Kato, N., *Bull. Soc. Japon. de Syph.*, 1931, **6**, 21.

⁵ Bessemans, A., and De Moor, A., *Bull. Soc. Franc. de Dermat. et Syph.*, 1937, **4**, 483.

⁶ Van Haelst, J., *C. R. Soc. de Biol.*, 1933, **113**, 1535.

⁷ Karrenberg, C. L., *Arch. f. Dermat. u. Syph.*, 1932, **165**, 585.

TABLE I.
Results Obtained by Kato³ in Darkfielding Nodes of Syphilitic Mice from One to 43 Days After Infection.

Days duration syphilitic infection	Treponemes in 250 darkfields per node	Days duration syphilitic infection	Treponemes in 250 darkfields per node
1	0	23	2
2	3	24	6
3	0	25	2
4	19	26	4
5	0	27	5
6	0	28	2
7	0	30	0
8	0	32	5
9	0	33	4
10	0	34	2
11	10	35	0
14	9	36	0
15	1	37	0
16	3	38	0
18	3	39	0
20	3	40	0
21	8	41	1
22	9	43	0

Total No. of mice, 36; total No. of Positive mice, 20.

would be only rarely necessary.

In this experiment, white mice were inoculated subcutaneously or intraperitoneally with Nichols rabbit strain of *Treponema pallidum*. Each mouse received 0.2 ml of rabbit chancre-emulsion containing more than 4 active treponemes per darkfield. A control rabbit inoculated with 0.5 ml of similar virus intratesticularly later developed a characteristic darkfield positive lesion.

The mice were kept in an air conditioned room at 65-70°F. They were housed in wire mesh cages with 4 to 10 mice per cage and were fed a proprietary food.† During the periods of observation, 45 and 90 days, unequivocal lesions of the mice were not observed. Forty-five and 90 days after infection, groups of mice were sacrificed and various organs from each mouse were emulsified in normal saline and inoculated into the testes of normal rabbits kept under similar conditions to the mice. The rabbits were examined at least twice weekly for the development of testicular lesions. Suspicious lesions were punctured and darkfielded. If characteristic motile treponemes were seen in the dark field, it was considered adequate proof that the mouse whose tissues had been subinoculated was infected with syphilis.

† Purina Laboratory Chow.

At the time these mice were sacrificed, iliac, axillary, or popliteal lymph nodes were dissected out and examined by Kato's method. The node was placed in a sterile petri dish and divided into 5 parts with a sharp scalpel. Each part was in turn placed in a drop of normal saline solution on a clean slide and emulsified by crushing and rotary movements of the flat surface of the scalpel blade. A cover slip was then placed on this emulsion, and 50 darkfields examined. A Spencer darkfield microscope with paraboloid condenser and built-in light was used. The objective was an oil immersion achromat with a numerical aperture usually of 0.8 being used together with a 10x ocular. Three observers examined the preparations. These all had considerable experience in recognition of the living *Treponema pallidum* by darkfield examination. All suspicious treponema-like objects were re-examined by the author.

The results as shown in Table II were completely negative. Because these results were obtained in an experiment mainly designed to study the biology of syphilis in the mouse, certain limitations existed which deserve discussion.

Actually, of the 36 mice employed, only 15 animals had darkfield examinations of a lymph node with simultaneous injections

TABLE II.

Comparison of the Results Obtained by Darkfielding Syphilitic Mouse Lymph Nodes with the Results Obtained by Inoculating Rabbit Testes with Mouse Tissues.

No. of mice	Route of infection	Duration of infection	Results of mouse organ*		Results of mouse node inoculation into rabbit testis		Darkfield study of node†
			inoculation into	rabbit testis	inoculation into	rabbit testis	
5	Subcutaneous	45 days	4/5	Positive	—	—	Negative
11	"	45 "	—	—	11/11	Positive	"
4	Intraperitoneal	45 "	4/4	Positive	3/4	"	"
6	"	45 "	4/6	"	—	—	"
10	"	90 "	9/10	"	—	—	"

* Either skin, blood, brain, spleen, or node, or a combination of these organs.

† Usually iliac node, rarely axillary or popliteal.

of other nodes into rabbit testes. Of these 15, none was infected intrascrotally, as Kato had done. Four of these 15 were infected by intraperitoneal injection, which is tantamount to intrascrotal injections in the mouse, which has free communication between the scrotum and the peritoneal cavity. It may seem from these statements that Kato's procedure was not duplicated. However, the important fact that 14 of the above 15 mice had chancrigenic nodes (for rabbits) suggests that the *Treponema pallidum* was present in the node that was simultaneously darkfielded. In addition, it is known from the work of many others⁸ that once any syphilitic mouse organ is shown virulent for rabbit testis, it is extremely likely that lymph nodes of the same mouse also con-

tain the syphilitic virus. From this last statement, it follows that the 33 mice with positive rabbit subinoculation tests most likely had nodes containing the *Treponema pallidum*, and that Kato's procedure of performing dark-fields was inadequate for demonstrating the organism. The existence of an invisible form of the syphilitic virus is not considered in this discussion.

Summary. 1. Attempts to use dark field examinations of the lymph nodes of mice as proofs of syphilitic infection 45 and 90 days after inoculation have failed, using the method employed successfully by Kato.

2. This failure to find the *Treponema pallidum* in lymph nodes by Kato's method confirms Karrenberg's studies.

3. More exhaustive methods of searching for the treponeme, such as those of Van Haelst, appear to be necessary.

⁸ Gueft, B., and Rosahn, P. D., *Am. J. Syph., Gon. and Ven. Dis.*, in press.

Canine Epilepsy Caused by Flour Bleached with Nitrogen Trichloride (Agene). I. Experimental Method.*

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(Introduced by A. Earl Walker.)

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Many reports on canine hysteria have stressed the remarkable resemblance of this syndrome to human idiopathic epilepsy. The nervous disorder in dogs is characterized clinically by irregular "grand mal" seizures, "running fits," ataxia, and "hysterical" states, with apparent recovery between attacks. The electro-encephalograms of these dogs show a cerebral dysrhythmia similar to that of human epilepsy. Because of these common features, it seems proper to term the condition *canine epilepsy*.

A dietary etiology of this disease has been proposed by various investigators, *e.g.*, gliadin toxicity,¹ lysine deficiency from wheat flour diet² and wheat gluten flour toxicity.^{3,4} This etiology is supported and explained by the crucial experiment of Mellanby.⁵ He showed that the treatment of flour with agene gas, *i.e.*, nitrogen trichloride (NCl_3), a process used on approximately 90% of the white wheat flour milled in both England and the

United States, rendered that flour convulsant to dogs. The toxicity of the wheat fractions used in the investigations cited above was presumably due to agene treatment (NCl_3 treatment)[†] of the flour source, inasmuch as untreated wheat fractions showed no toxicity. The purposes of this communication are to describe a method of producing consistently and rapidly a convulsive disorder in dogs, and to delimit the toxic principle.

Method. A diet having a high flour component was prepared. The control ration, including supplementation with accessory food substances, and the actual analysis of this diet, is given below.

The diet, with water added, was baked into biscuits at 395°F for 30 minutes. The biscuits were canned and pasteurized after sealing. The analysis shown in Table I was made of these biscuits 2 weeks after baking.

The experimental ration was identical except that the flour component was heavily treated with agene. This was done in a bleaching unit in which the gas is generated by the interaction between chlorine gas and 1% ammonium chloride solution at a pH of 3.0. The NCl_3 gas thus formed was blown over the flour by means of an air stream, and was applied as a 1% solution of NCl_3 in air saturated with water vapor. Agene gas was used as the bleaching agent for all experimental flour samples, in a concentration of 30 g per 100 lb of flour, this value being determined by controlling the amount of chlorine gas available for reaction. No attempt was made to determine how much of the NCl_3 gas evolved had reacted with the flour.

* We are indebted to the following: Messrs. T. Soloski, J. Cryns, C. McWilliams, and Miss A. Felsher of the Cereal and Baked Products Branch of the Food and Container Institute for baking the diets used in this investigation; Messrs. S. Bishov, M. Wolf, M. Rolland, and Miss M. Graham of the Chemistry Division for aid in fractionating the flour and chemical analyses; T/5 Ellis O. Jones for technical assistance.

¹ Melnick, D., and Cowgill, G. R., *J. Nutrition*, 1937, **14**, 401.

² Arnold, A., and Elvehjem, C. A., *J. Am. Vet. Med. Assn.*, 1939, **95**, 303.

³ Wagner, J. R., and Elvehjem, C. A., *J. Nutrition*, 1944, **28**, 431.

⁴ Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 115.

⁵ Mellanby, E., *Brit. Med. J.*, 1946, Dec. 14, No. 4484, 885.

[†] The word "agene" is used throughout interchangeably with nitrogen trichloride gas, NCl_3 .

TABLE I.
Basic Diet Used in Producing Canine Epilepsy.

Composition		Analysis	
	%		%
Casein	5	Protein	17.5
Liver powder	5	Carbohydrate	65
Salts IV	4	Fat	5
Sucrose	6	Ash	4
Corn oil	5	Moisture	8.5
Flour	75		
	100		100
Supplementation			
	mg/kilo		mg/kilo
Thiamine	5.6	Thiamine	4.3
Riboflavin	5.6	Riboflavin	10
Pyridoxine	4		
Niacin	60	Niacin	94
Pantothenic acid	15		
Para amino benzoic acid	15		
Inositol	15		
Ascorbic acid	80	Ascorbic acid	120
Haliver oil	500		
α -tocopherol	100		

Results. When the control ration was fed to dogs of 5 to 10 kg weight, in quantities of 250 to 300 g per day, they gained weight, had a good appetite, and showed no symptoms of neurologic disorder or deficiency disease in the periods up to 45 days during which they were observed.

When the experimental diet (bleached flour) was fed to dogs, regardless of age, sex or the breed used, all the animals developed convulsions within 5 days, together with marked behavior disturbances and an ataxic gait. The electroencephalogram showed marked abnormalities, appearing between the 2nd and 3rd day of the experimental diet. No changes appeared after 45 days on the control diet. In all, 24 dogs were fed the experimental diet, and all 24 dogs developed canine epilepsy.

The wheat flour was fractionated after trials with numerous fat solvents showed that Bloor's mixture (75% anhydrous ethyl alcohol, 25% di-ethyl ether) was most satisfactory. By repeated extractions, it was possible to remove 1.4% fat from a straight grade of hard red winter wheat, the flour thus obtained containing less than 0.15% of fat by analysis. The solvent was removed from the fat at 100°C and a pressure of 20 mm Hg with a counter current of nitrogen,

and both fat free flour and fat, were treated with agene. Feeding experiments showed that the toxicity remained unimpaired in the flour fraction, while the fat (although it became dark and rancid) did not prove toxic to test animals. Separation of gluten from the fat free flour showed that convulsive activity was retained in the protein fraction. Further confirmation of the relation between toxicity and the protein fraction is offered by the fact that casein treated with agene proved convulsant to 4 dogs.

The extraction of fat is emphasized, because commercially produced gluten has the lipid so firmly bound that it is not fully extractable at this stage. Hard red winter, hard red spring, and soft red spring wheat of clear, straight and patent grades all produced convulsions after treatment with agene although straight grade (so-called "family-grade") of hard red winter wheat was used in most of these experiments.

Monkeys were also used. The control diet seemed adequate to maintain nutrition in 6 monkeys for 3 weeks. The experimental diet did not produce convulsions, but the monkeys developed a tremor of the extremities and weakness of the hind limbs, within 5 days after changing to the experimental diet. This syndrome persisted 6 weeks later.

Canine Epilepsy Caused by Flour Bleached with Nitrogen Trichloride (Agene). II. Role of Amino Acids.*

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Epilepsy in dogs fed bleached flour is attributable to the toxic effect of wheat proteins modified by nitrogen trichloride.^{1,2} The heat stability of the toxic material, its presence in agenized gliadin and casein, and the rapid onset of symptoms² suggest that the toxicity resides in a relatively small molecule, possibly an altered amino acid. Thus it seemed advisable to test amino acids treated with nitrogen trichloride for convulsant properties.

The results of such tests in control and epileptic dogs are reported in the present work, changes in electroencephalographic pattern being used as a measure of convulsant activity. The electroencephalograms of the epileptic dogs prior to administration of amino acids also provide further insight into the syndrome.

Method. A mixture of synthetic amino acids was prepared to resemble the composition of gliadin. One-half of this amino acid mixture was kept as a control, and the other half was "bleached," *i.e.*, treated with agene (NCl_3) in the same manner as the flour.² The mixed amino acids were exposed for 10 minutes to 1% NCl_3 in air saturated with water vapor. No analysis for lost excess gas was performed. 3.3% solutions of the amino

acid mixtures were prepared by adjusting aqueous suspension to pH 10 with 10N NaOH, and then adjusting the pH to 8.5 with concentrated HCl. They were then used for intravenous injection.

Dogs were prepared under vinyl ether for artificial respiration, and for intravenous infusion *via* the femoral vein. The electrical activity of the brain and the electrocardiogram were recorded by means of Grass amplifiers and inkwriters. Paralysis was initiated by the intravenous injection of 25 mg dihydro- β -erythroidine hydrobromide and maintained by infusion of the erythroidine (1 mg per ml in 0.156 M sodium chloride). Under artificial respiration, the vinyl ether was rapidly eliminated and within 15 minutes the electroencephalogram resembled that of the intact animal. The dog was now available for testing the convulsant properties of the amino acid mixtures.

Results. Eight dogs on the experimental diet containing agenized flour (*i.e.*, bleached flour) for periods up to 2 weeks were used. These showed abnormal electroencephalograms but no seizures during a pre-injection period of 1 to 2 hours. The pattern illustrated in Fig. 1b is characteristic of all dogs on the experimental diet, and resembles that reported by others.³

Seizures followed the intravenous administration of the agene-treated amino acid mixture in less than 5 minutes (Fig. 1d, e, f). The absolute amount of amino acids varied

* The bleaching equipment was supplied through the kindness of the Wallace and Tiernan Company, Chicago. The synthetic amino acids and β -erythroidine were supplied through the kindness of Merck and Co., courtesy of Dr. J. M. Carlisle.

¹ Mellanby, E., *Brit. Med. J.*, 1946, Dec. 14, No. 4484, 885.

² Silver, M. L., Zevin, S. S., and Johnson, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **63**, 408.

³ Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 115.

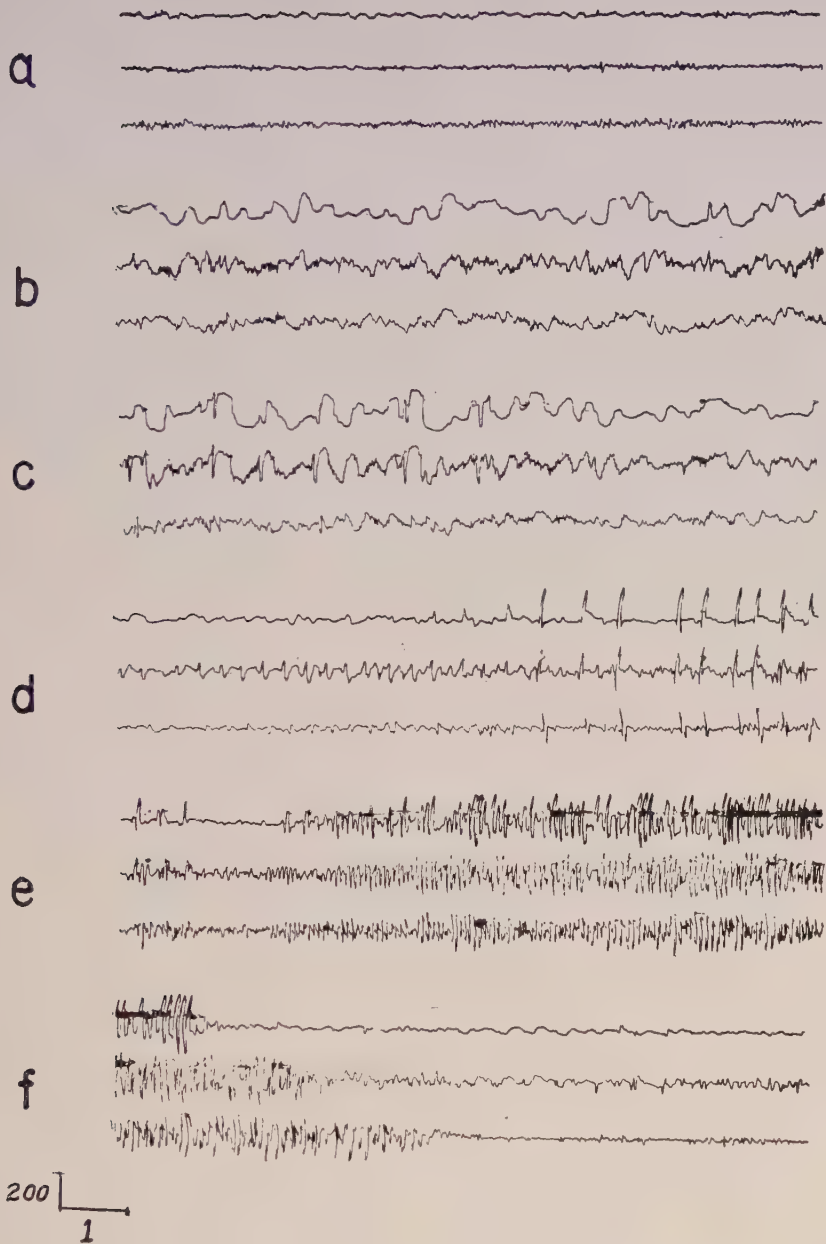


FIG. 1.

a. Normal electroencephalogram of dog on control (unbleached) diet. The upper lead is left parietal to ground; the middle lead is right parietal to ground; the lower lead is right parietal to left parietal (bipolar); this is constant in all tracings.

b, c. Abnormal E.E.G. of dog fed experimental (bleached) diet for three days. Note resemblance to petit mal activity.

d, e, f. In same dog as above, continuous record of a grand mal seizure occurring less than 5 minutes after the intravenous injection of "bleached" amino acid mixture. In small figure at lower left, voltage calibration is 200 μ V, time calibration is 1 second. Each frame is ten seconds.

from 0.2 to 1.5 g. The injection of control untreated amino acids was ineffective in 4 dogs even after the injection of 20 g in 120 minutes.

In 12 dogs which were on the control diet containing untreated flour (*i.e.*, unbleached flour), the intravenous administration of the agene-treated amino acid mixture was followed by seizure patterns in the electroencephalogram of 4 dogs, marked abnormalities in 3 dogs, and no significant change in 5 dogs. In the animals with abnormal E.E.G.'s produced by intravenously administered agenzized amino acids, the intracisternal injection of 1 to 2 cc of the NCl_3 -treated amino acid mixture was followed by seizures within 5 minutes after injection.

Experiments have been conducted with individual amino acids treated in their crystalline states and the results have been negative

for arginine, glutamic acid, glycine, histidine, lysine, methionine, tryptophane and tyrosine; equivocal for proline, serine, and aspartic acid; and positive for cysteine hydrochloride. A possibility that should not be overlooked is that the toxic compound in foodstuffs may be an altered polypeptide.

Summary. When an amino acid mixture approximating the composition of gliadin is treated with nitrogen trichloride gas (NCl_3) this mixture is so altered as to make it convulsant for dogs when given intravenously. The convulsant activity is most readily demonstrated in dogs which have been on a bleached (agenized) flour diet for 5 to 7 days, presumably because these animals have accumulated sufficient quantity of the toxic material *via* the alimentary tract, to make them highly susceptible to an additional increment received *via* the blood stream.

16108

Adrenal Cortical Activity in Urine of Horses.

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The presence of a substance in urine that protects adrenalectomized rats from the stress of cold was first noted by Anderson, Haymaker and Joseph¹ and Weil and Browne.² More extensive investigations have shown that extracts of urine from normal human subjects when tested in adrenalectomized rats

cause an elevation of liver glycogen,^{3,4} increased life maintenance,^{3,5} prolongation of muscle work performance,⁶ and prevention of water intoxication.⁷ Although the above studies have employed extracts of human urine, Selye and Dosne⁸ have reported that the urine of large domestic animals contains cortin-like activity. The purpose of this in-

¹ Anderson, E., Haymaker, W., and Joseph, M., *Endocrinology*, 1938, **23**, 398.

² Weil, P., and Browne, J. S. L., *Science*, 1939, **90**, 445.

³ Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *J. Biol. Chem.*, 1943, **148**, 455.

⁴ Horwitt, B. N., and Dorfman, R. I., *Science*, 1943, **97**, 337.

⁵ Dorfman, R. I., and Horwitt, B. N., *Fed. Proc.*, 1943, **2**, 60.

⁶ Shipley, R. A., Dorfman, R. I., and Horwitt, B. N., *Am. J. Physiol.*, 1943, **139**, 742.

⁷ Schiller, S., and Dorfman, R. I., *Endocrinology*, 1943, **33**, 402.

⁸ Selye, H., and Dosne, C., *Lancet*, 1940, **239**, 70.

TABLE I.
Liver Glycogen Response of Horse Urine Extracts.

Liver glycogen					
Sample No.	Avg urine equiv. cc	Negative controls mg/100 g liver weight	Urine extract	Percentage increase	Remarks
				%	
Total extracts.					
B-18	200	37 (40)*	44 (22)*	+19	Normal urine
38-191	"	33 (35)	38 (19)	+15	Pregnant mare
176-10	"	45 (19)	53 (10)	+18	" "
Ketonic fraction					
38-174	400	40 (12)	80 (9)	+100	Normal urine
38-175-2	"	80 (10)	90 (10)	+ 12	" "
38-187	250	130 (15)	190 (10)	+ 46	" "
38-191-1	330	30 (20)	100 (15)	+230	Pregnant mare
176-5-1	250	30 (10)	80 (10)	+170	" "
Non-ketonic fraction.					
38-174	200	60 (10)	60 (3)	0	Normal horse
38-175-2	"	90 (15)	60 (9)	—33	" "
38-187	"	90 (15)	50 (9)	—45	" "
38-191	"	30 (15)	30 (9)	0	Pregnant mare

* Numbers in parentheses refer to number of mice employed.

vestigation was to assess the concentration of cortin-like activity in the urine of horses.

Method. The assay employed was a modification of the methods used by Venning, *et al.*⁹ and Eggleston, *et al.*¹⁰ Male white mice of the Swiss-Webster strain from our own stock colony weighing 20-25 g were used. At least a week before being used the mice were placed on a purified diet which contained 25% protein, 48% carbohydrate, 25% fat and 2% salt mixture,¹¹ plus adequate vitamin supplements. The animals were anesthetized with ether and adrenalectomized by the usual lumbar route. Following adrenalectomy they were placed in a warm room and given the above diet and 0.9% NaCl in place of their usual drinking water. On the morning of the fourth postoperative day food was removed and each mouse was given 25 mg glucose orally, and the first of 7 hourly injections of the extract under test. One hour after the last injection the mice were weighed and anesthetized with nembutal. The livers were quickly removed, weighed

and placed in hot 30% KOH for digestion. Glycogen was precipitated with alcohol and after hydrolysis, total reducing substances were determined by the method of Good, Kramer and Somogyi.¹² Total liver glycogen has been expressed as milligrams of glucose per 100 g of liver weight.

Urine Extracts. Urine was collected under toluene and acidified with acetic acid. All urines were extracted 3 times with one-quarter volume of ethylene dichloride or chloroform, usually employing a motor stirrer for the extraction. The combined extracts were then evaporated almost to dryness under reduced pressure and taken up in small amounts of 95% ethanol and diluted for injection or further fractionated by the method described by Venning, *et al.*¹³

Results. Table I lists the results obtained with 3 different types of extracts. Crude urine extracts that were not fractionated are referred to as total extracts. The ketonic and non-ketonic fractions were obtained by the Girard's reagent separation procedure.¹³ Many of the total extracts of horse urine that have been prepared in this laboratory have shown evidence of toxic substances that resulted in a decrease in liver glycogen below the level of the negative controls. Dorfman,

⁹ Venning, E. H., Kazmin, V. E., and Bell, J. C., *Endocrinology*, 1946, **38**, 79.

¹⁰ Eggleston, N. M., Johnston, B. J., and Dobriner, K., *Endocrinology*, 1946, **38**, 197.

¹¹ Bosshardt, D. K., Ciereszko, L. S., Buffington, A. C., and Arnow, L. E., *Arch. Biochem.*, 1945, **7**, 1.

¹² Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

¹³ Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *Endocrinology*, 1944, **35**, 49.

*et al.*¹⁴ Venning, *et al.*³ have commented on this toxic effect found in extracts of human urine. Fractionation evidently removes some of the toxic activity since glycogen responses are higher in the ketonic fraction than in the total extracts. As was to be expected no evidence of cortin-like activity was found in the non-ketonic fractions.

As a standard preparation for comparison, Wilson's aqueous adrenal cortical extract has been used. Comparing liver glycogen responses to Wilson's extract with the average response of the ketonic fraction of the horse urine extracts it was calculated that 1 liter of normal horse urine contains an equivalent of 0.10-0.15 cc of Wilson's extract and 1 liter of pregnant mare urine contains an equivalent of 0.30-0.40 cc of this extract. These figures compare favorably with results of Dorfman *et al.*¹⁵ and Venning *et al.*³ on human urine

extracts but appear to be definitely lower than the results reported for horse urine by Selye and Dosne.⁸ The quantitative assay of the concentration of cortin-like material in urine is complicated by so many factors that the results presented here should be considered only as estimations.

Summary. The presence of cortin-like activity in extracts of horse urine has been confirmed. It is estimated that the concentration of this active material is approximately the same in urine of normal horses and normal humans. There is some indication that urinary cortin-like activity is increased in pregnancy.

¹⁴ Dorfman, R. I., Shipley, R. A., Schiller, S., and Horwitt, B. N., *Endocrinology*, 1946, **38**, 165.

¹⁵ Dorfman, R. I., Horwitt, B. N., and Fish, W. R., *Science*, 1942, **96**, 496.

16109

Nutrition of the Mouse. IV. Comparison of Bacterial Population of Two Highly Inbred Strains.*

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It was shown by Rogers, McElroy and Cowgill¹ that the nutritional requirements for reproduction and lactation differed for 2 strains of mice. Fenton and Cowgill² showed

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We wish to acknowledge gifts of B-complex vitamins from Merck and Company, Inc., Hoffmann-LaRoche, Inc., and Lederle Laboratories, Inc. The technical assistance rendered by Marie A. Stone was greatly appreciated.

¹ Rogers, L. K., McElroy, L. W., and Cowgill, G. R., *Science*, 1942, **95**, 203.

² Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **34**, 273.

the existence of a strain difference with respect to the riboflavin requirement for growth. Unpublished work in this laboratory has shown that this is also true of the pantothenic acid requirement. This dissimilarity of the nutritional requirements of 2 strains of mice may in part be due to different rates of intestinal synthesis of essential nutrients. The cecum has been thought to be an important site of the synthesis of B vitamins.^{3,4,5,6} Man-

³ Guerrant, N. B., Dutcher, R. A., and Tomey, L. F., *J. Biol. Chem.*, 1935, **110**, 233.

⁴ Taylor, A., Pennington, D., and Thacker, J., *U. of Texas Pub.*, 1942, **4237**, 135.

⁵ Schweigert, B. S., McIntire, J. M., Henderson, L. M., and Elvehjem, C. A., *Arch. Biochem.*, 1945, **6**, 403.

⁶ Mannering, G. J., Orsini, D., and Elvehjem, C. A., *J. Nutrition*, 1944, **28**, 141.

TABLE I.

The Weight of the Cecal Contents and the Bacterial Count per Gram of Cecal Contents and per Cecum of the 2 Strains of Mice.

Strain	Diet	No. of animals	Wt of cecal contents, g	Bacterial count	
				per cecum, billions	per g of cecal contents, billions
A strain	101	12	.065 ± .021*	1.5 ± .84	22.2 ± 11.5
C ₅₇ strain	101	12	.176 ± .047	5.11 ± 2.62	28.7 ± 11.5
Young A strain	stock	3	.386 ± .038	13.12 ± 3.03	32.4 ± 4.34
Young C ₅₇ strain	"	3	.352 ± .070	8.39 ± 1.76	23.9 ± 2.1
Older A strain	"	6	.417 ± .045	19.86 ± 6.1	48.0 ± 16.8
Older C ₅₇ strain	"	3	.406 ± .125	15.1 ± 5.9	37.0 ± 13.4

* Standard deviation.

nering *et al.*⁶ worked with rats fed synthetic diets containing various carbohydrates and state that with certain exceptions the riboflavin found in the feces was related to the dry weight of the cecal contents.

The purpose of this investigation was to study the intestinal flora of 2 strains of mice, one with characteristically high susceptibility to spontaneous mammary tumors (strain A) and the other with low susceptibility in this respect (strain C₅₇). These two strains were chosen because they had been used in previous studies in this laboratory.

Experimental. The 39 mice used in this study had been maintained on one of 2 diets: a synthetic ration containing dextrose as the carbohydrate without added biotin or folic acid (diet 101 as described previously⁷) or a stock ration.[†] Twelve mice of the C₅₇ strain (tumor-resistant) and 12 of the A strain (tumor-susceptible) ranging in age from 2 to 4 months were used after having been maintained on the synthetic ration. The remainder of the animals had been fed the stock diet and fell into 2 age groups, namely, 2-2.5 months and about 12 months old. The mice were sacrificed by etherization and the cecal contents removed and weighed. Bacterial counts and cultural work were carried out as described in another publication.⁸

Results. As shown in Table I, the mice of the A strain on the synthetic diet had smaller

cecal contents than the C₅₇ mice on the same diet. In only one case did the highest value given by an A strain mouse equal the lowest weight obtained from a C₅₇ animal. The differences in weights of cecal contents between these 2 groups was found to be statistically highly significant ($p < 0.01$). The total bacterial count per g of cecal contents was about the same in both strains. However, the effect of the larger cecal contents of the black mice was reflected in the greater total bacterial population of the cecum of this strain when compared to the white animals. This difference was also found to be statistically highly significant ($p < 0.01$).

The results obtained by studying the Gram stains and the cultures isolated from the ceca of both strains were similar with respect to the occurrence of cocci and Gram-positive rods. However, there appeared in the highest dilutions of the C₅₇ mice in 10 cases out of 12 a short Gram-negative, anaerobic rod. This type of organism occurred in only 2 white mice out of 12. Coliform organisms were encountered slightly more frequently in high dilutions of cecal contents of the A strain mice (5 out of 12 animals) than in the C₅₇ strain (2 out of 12 mice).

These two strains of mice, when fed a stock ration, showed no differences in weights of cecal contents and total bacterial population per cecum. The lower values shown by C₅₇ mice for bacterial counts per g of cecal contents and per cecum were not considered significant owing to the wide variation in counts and to the small number of animals in each series. On microscopic examination of the

⁷ Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **33**, 703.

[†] Purina Laboratory Chow.

⁸ Gall, L. S., Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, in press.

Gram stain and from cultural results the bacterial flora of the cecum of the older animals of both strains seemed to be similar, but there was a tendency for the younger black animals (C₅₇) to have a more varied flora than was shown by the white mice of the same age. It is unsafe to draw a general conclusion from so small a series of mice, even though this difference was observed on all animals.

In Table I the values for the stock ration animals are broken down into groups of young and older mice, so that these stock animals may be compared with those on diet 101. As was pointed out in a previous paper by these authors,⁸ the weight of the cecal contents and the bacterial count per g and per cecum were considerably higher in the A strain mice on the stock diet than in the A strain mice fed a synthetic ration. By inspection of the data in Table I, it is evident that even though the black animals fed a synthetic diet had greater cecal contents and higher bacterial counts per cecum than the A strain mice, they still fell short in these respects of the C₅₇ animals of the same age on the stock diet.

Discussion. There has been no really satisfactory explanation offered for the strain differences in requirement for certain of the B vitamins, as manifested by mice of the C₅₇ and A strains. (Grüneberg⁹ discussed the various anatomical and physiological differences in the alimentary tracts of mice of several strains, but there is no mention made of any variations in the amount of cecal contents, or in the size of the cecum. It is therefore of interest to note that in our study a significant difference in the weight of the cecal contents of 2 strains of mice was found when these animals were fed a synthetic diet, especially since the A strain mice, which had the smaller cecal contents also had a higher dietary requirement for certain B vitamins.

It has been shown^{3,4,5} that the cecum is one of the sites of bacterial synthesis of B vitamins in the intestinal tracts of rats. For this reason it may well be that the larger bacterial population offers an explanation for the lower requirements of the C₅₇ strain for certain B vitamins. Further support for this theory is found in the cultural work done on the cecal contents of animals fed this synthetic diet in which it was demonstrated that bacteria isolated from top dilutions of these contents in numbers of 100 million or more per gram were able to synthesize some of the B vitamins *in vitro*.¹⁰

Summary and Conclusions. 1. Studies were made to determine whether there is a difference in the weight and bacterial population of the cecal contents of mice of the C₅₇ and A strains, which were the same age and received the same diet.

2. The C₅₇ animals fed a synthetic diet (No. 101) had a significantly greater amount of cecal contents and bacterial count per cecum than the A strain mice of the same age and fed the same diet. Although most of the bacterial flora appeared to be the same in the 2 strains, a Gram-negative, anaerobic rod did occur more frequently in the C₅₇ mice than in the A strain.

3. There was no significant difference between the black and white mice of the same age fed the stock ration with respect to weight of cecal contents or bacterial count per cecum. The young black mice may have had a more diverse flora than the white mice of the same age.

4. It was postulated that this greater weight of cecal contents with its higher bacterial population in the C₅₇ mice might help to explain the lower B vitamin requirements of this strain when comparing it with animals of the A strain, both fed the same synthetic diet.

⁹ Grüneberg, Hans, *Genetics of the Mouse*, 1943, Cambridge University Press, London, England.

¹⁰ Gall, L. S., Illingworth, B. A., Cowgill, G. R., and Fenton, P. F., *J. Nutrition*, in press.

Spasmolytic Action of Dolophine (Amidone).*

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(Introduced by Nilkanth M. Phatak.)

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Amidone (6 dimethylamino-4, 4, diphenyl-3-heptanone) is a new synthetic analgesic compound related to demerol (Dolantin) originally prepared at the I.G. Farbenindustrie research plant at Höchst am Main and first described in this country in the U. S. State Department Technical Intelligence Team Report.¹ Scott and Chen² reported that this agent had marked analgesic properties when tested in both dogs and humans by the Wolff-Hardy technic as well as a comparatively low toxic/analgesic ratio. Later, these workers named the compound "dolophine" and reported the clinical trial of 4 related compounds.³ They also found that "dolophine" possessed about equal spasmolytic action when compared with demerol on isolated intestinal strips. Karr⁴ found decreased propulsive activity of the intestines of rats and rabbits after "dolophine" which was slightly more marked than the decrease noted after demerol. We have further studied the action of these spasmolytic agents on isolated intestinal strips and, in addition, have compared their effects on the isolated uterine musculature of rabbits and rats.

Method. The usual technic for recording contractions of isolated muscle strips was used. Furfuryl trimethyl ammonium iodide (Furmethide)⁵ served as the spasmogenic

agent for the rabbit intestinal strips and the rat uteri. Methyl ergonovine (Methergine)⁶ was used to stimulate contraction in rabbit uterine strips. Other agents were also used for stimulating contraction.

Results. "Dolophine" and demerol produced about the same degree of spasmolysis on the isolated rabbit intestine when equal concentrations of the drugs were used, with concentrations as low as 0.025 mg/50 cc (1:2,000,000) being effective. These results for demerol agree with other reports.^{7,8,9}

The contracted rat uterus showed a similar relaxation when concentrations of either drug as low as 1:2,000,000 were used. Rabbit uteri, however, required more "dolophine" than demerol for spasmolysis on most trials, the least effective concentration being 0.075 mg/50 cc (1:666,666). Normally active smooth muscle strips were found to respond irregularly to both drugs.

The pharmacodynamic basis for the spasmolytic action of these compounds is not fully known. Since "dolophine" lysed spasm induced by both musculotropic (Methergine, Barium, Pitressin) and neurotropic (Furmethide, Neostigmine, Acetylcholine) spasmogenic agents, its action is probably largely musculotropic. In this respect it resembles trasentin, which, in addition to a musculotropic spasmolytic action, shows some local anesthetic properties. A recent report¹⁰ in-

* Supported by a grant from the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana. We are indebted to Dr. Kohlstaedt and Dr. C. C. Chen for the dolophine used in this study.

¹ Kleiderer, E. C., Rice, J. B., Conquest, V., and Williams, J. H., Report 981, Office of the Publication Board, Department of Commerce, Washington, D.C.

² Scott, C. C., and Chen, K. K., *J. P. E. T.*, 1946, **87**, 63.

³ Scott, C. C., Kohlstaedt, K. G., and Chen, K. K., *Anesth. and Analges.*, 1947, **26**, 12.

⁴ Karr, N. W., *Fed. Proc.*, 1947, **6**, 343.

⁵ Fellows, E. J., and Livingston, E. A., *J. P. E. T.*, 1940, **58**, 231.

⁶ Kirchhof, A. C., Racely, C. A., Wilson, W. M., and David, N. A., *West. J. Surg., Gyn. and Obs.*, 1944, **52**, 197.

⁷ Yonkman, F. F., Noth, P. H., and Hecht, H. H., *Ann. Int. Med.*, 1944, **21**, 7.

⁸ Climenko, D. R., *Fed. Proc.*, 1942, **1**, 15.

⁹ Yonkman, F. F., *Anesth. and Analges.*, 1944, **23**, 207.

¹⁰ Way, E. L., *Science*, 1945, **101**, 566.

icates that demerol, like trasentin, has local anesthetic properties and Everett¹¹ in this laboratory has demonstrated that "dolphine" produces local anesthesia in the rabbit's cornea. Thus, the local anesthetic property, as well as musculotropic spasmolysis seems to be common to trasentin, demerol and "dolphine."

¹¹ Everett, F. G., personal communication.

Summary. Isolated strips of rabbit intestine and rat and rabbit uteri respond with spasmolysis when treated with "dolphine" and demerol when spasm is induced with furmethide or Methergine. The ratio of spasmolytic concentration is approximately 1:1 although rabbit uteri required slightly more dolphine than demerol for spasmolysis.

16111 P

Induction of Male Copulatory Behavior in the Brown Leghorn Hen.*

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Previous experiments have shown that daily injections of testosterone propionate in poulards¹ and pullets² will induce masculinization of somatic sex characters and behavior. These individuals crowed, became combative, and displayed male courtship but no copulatory behavior, even though injections were continued for periods up to 7 months. The absence of this phase of behavior has long baffled investigators in view of the ease with which other sexual characters may be masculinized in the fowl. Two incidental cases are recorded^{3,4} in which hens revealed male copulatory behavior, although neither instance is based on extensive experimentation. The present experiments, in progress for over 4 years, show that masculine copulatory behavior may be induced and maintained in some pullets following suc-

cessive implantations of androgen pellets.

Each of 4 non-laying pullets (A, BW, G, and GA) received subcutaneous implantations of testosterone propionate pellets (average weight, 33.4 mg) when 5 months old and subsequently at 84, 164, 244, 337, 425, 530, 630, and 727 days. Two control pullets (B and W) received implantations of cholesterol pellets averaging 32.6 mg. Pellet implantations were repeated upon absorption, determined by palpation and comb regression. The behavior was tested, generally on alternate days, by introducing each bird, alone in its pen, to a female, to a male, or to a dummy female mounted in the squatting, copulatory position.

Experimental pullet A was first observed to crow at 34 days, to chase, grab, and attempt mounting at 95 days, to grab, mount, and copulate at 110 (age 262), and to waltz and circle at 114 days after the first implant. Pullet BW was also first observed to crow at 34, chase, grab, and attempt mounting at 95, mount and copulate at 103 (age 255), and waltz and circle at 127 days. A continued to copulate for 5 months, when it died, and BW for 8 months after the final implant. Courtship activity preliminary to copulation, never very vigorous, was frequently absent. In no instance did they indicate receptivity by squatting at the approach of the male.

* This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago. Grateful acknowledgment is made to Dr. Erwin Schwenk of the Schering Corporation for the testosterone propionate used in these experiments.

¹ Davis, David E., and Domm, L. V., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 667; *Essays in Biology*, University of Calif. Press., 1943, 171.

² Domm, L. V., Davis, David E., and Blivaiss, Ben B., *Anat. Rec.*, 1942, **84** (Suppl.), 31.

³ Domm, L. V., *J. Exp. Zool.*, 1927, **48**, 31.

⁴ Zitrin, A., *Endocrinology*, 1942, **31**, 690.

The other pullets (G and GA) displayed courtship but no masculine copulatory behavior during the period of observations.

In the second group, 6 pullets received their first pellet at one month and further implants (average, 25.9 mg) at 109, 202, 297, 395, 495, and 592 days thereafter. One bird, G, was first observed to crow at 125, chase at 126, waltz and circle at 141, and to mount and copulate at 161 days (age 191) after the first implant. Sporadic copulations occurred until 11 months after final implantation. The other 5 birds began to crow and display courtship at approximately the same time as G but did not mount or copulate.

In a third group, 8 pullets received pellet implantations beginning at 4 days and additional implants (average, 23.2 mg) at 62 and 172 days. In this group, only one bird crowed and another was observed to waltz and circle.

In the fourth group, 6 pullets received the first pellet when 3 months old and further implants (average, 28.2 mg) at 80, 185, 317,[†] and 436 days subsequently. All were known to crow and 3 displayed courtship behavior. No copulations were observed.

A standard trap-nest was kept in all pens throughout the experiments. One treading pul-

[†] At the time of the second, third, and fourth implantations, 3 of the pullets received 2 pellets each. The average weight of both was 57.1 mg.

let (A) never laid. It died 871 days after first implantation. The other treader (BW) laid 6 eggs between the 69th and 80th days following second implantation, and 3 more after a lapse of one year following the last implant. During the 4 years of observation the non-treading pullet G laid 6 eggs between 57th and 74th days, and GA only one egg on 79th day after second implantation.

In the second experiment, treading hen G, as well as 3 non-treaders, never laid over a span of 3 years. One non-treading hen laid an egg at 4 months after the first, and another laid one egg at 2 years after the last implant. Similar records of sporadic egg-laying were obtained in the other experiments, while some controls laid as many as 21 eggs per month.

Summary. Three cases are described from 4 experimental groups totaling 24 birds, some observed for more than 4 years, in which single comb brown Leghorn hens displayed male copulatory behavior following successive implants of testosterone propionate pellets. Implantations were begun when birds were 4 days, and 1, 3, and 5 months old. These results contrast with earlier negative findings following single daily injections of this hormone. Induction of copulatory behavior in such a small percentage of cases indicates the desirability for further study of factors controlling this behavior pattern.

16112

Incidence of Rh Factor Among the Indians of the Southwest.

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Within the last 12 years the Indians of the Southwest have been investigated as to the occurrence of the various agglutinogens. Allen and Schaeffer¹ reported on the classic blood groups, and Allen and Larsen² reported their

findings on the M and N agglutinogens. The basis of this report is the Rh factor among the above mentioned Indians.

* Junior Research Fellow from the National Institute of Health.

¹ Allen, F. W., and Schaeffer, W., *Univ. N. Mex. Bull. Biol. Series*, 1935, **4**, 2.

² Allen, F. W., and Larsen, H. D., *J. Immunol.*, 1937, **32**, 301.

The Indians tested were patients at the U. S. Indian Hospital and the Indian School Hospital at Albuquerque, New Mexico. Students of the Indian School at Albuquerque and the Day School at Isleta were also used in this study. The age range was from school age to 60 years.

Lederle's Anti-Rh Serum (Anti-Rh₀) was used in this series of tests; this serum is of animal origin. Two drops of blood to be tested were added to 0.5 cc of physiological saline containing 1% sodium citrate. One drop of the cell suspension is placed on a clean glass slide. Next to this is placed one drop of Anti-Rh Serum. The two are mixed with a glass rod and finally by tilting the slide back and forth once. The slide is placed undisturbed on the table for 3 minutes. At the end of this time the slide is picked up and given 2 or 3 gentle turns. While continuing to hold the slide a gentle motion is maintained and the test mixture is examined macroscopically with oblique lighting until the end of 6 minutes. Clumping is always visible within 6 minutes in positive tests and all observations must be terminated in 6 minutes. It is necessary to guard against rapid evaporation in dry air; a Petri dish was used to cover the slides. Rh-positive and Rh-negative controls were run with all tests.

The census records of the United Pueblo Agency at Albuquerque were used to determine the racial purity of the Indians concerned. Table I records the findings of the full-blooded (4/4) Indians tested.

Seven Rh positives were found among Indians other than full bloods (4/4). The range of racial purity ran from 2/4 to 15/16.

TABLE I.
Incidence of Rh Factor Among Full-Blooded (4/4)
Indians Tested.

Tribe or Pueblo	Rh positive	Rh negative
Acoma	62	0
Apache	8	0
Cochiti	6	0
Hopi	6	0
Isleta	75	0
Jemez	9	0
Laguna	70	0
Navajo	53	1
Sandia	6	0
San Felipe	22	0
San Ildefonso	1	0
San Juan	6	0
Santa Ana	10	0
Santa Clara	3	0
Santo Domingo	19	0
Sia (Zia)	8	0
Taos	12	0
Tesuque	3	0
Tewa	1	0
Zuni	5	0
Totals	385	1

In some cases census records were unavailable; there were 55 Indians in this category. All were Rh positive.

The total findings are 447 Rh positives and one Rh negative in the Indians tested.

Similar results have been obtained by other investigators^{3,4} in testing Indians of North America.

The author wishes to express his indebtedness to Martin W. Fleck, of the Biology Department at the University of New Mexico, for his critical suggestions during the progress of this problem, and Julia Fritz Gerheim for her technical assistance.

³ Landsteiner, K., Wiener, A. S., and Matson, G. A., *J. Exp. Med.*, 1942, **76**, 73.

⁴ Wiener, A. S., Zepeda, J. P., Sonn, E. B., and Polivka, H. R., *J. Exp. Med.*, 1945, **81**, 559.

Chemistry of Melanin. IV. Electron Micrography of Natural Melanins.*

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Although natural melanins apparently consist of insoluble, heterogeneous aggregates^{1,2,3} a water-soluble melanin-containing pseudoglobulin has been separated from pigmented tissue.⁴ In this study, we have examined both melanin granules and the melano-pseudoglobulin with the electron microscope to determine (a) whether melanin consists of formed elements or amorphous aggregates, (b) whether structure upon the surface of and within the granule is discernible, and (c) what formal relationship exists between melano-pseudoglobulin and the natural pigment.

Experimental and Results. Electron micrographs were taken at a magnification of 7,600 diameters with a Universal model (R.C.A.) electron microscope. Collateral optical microscopy was carried out with a Zeiss phase contrast microscope† at a magnification of 1085 diameters. Samples of 50 granules were measured to determine size ranges.

Melanin granules from the S-91 transplantable mouse melanoma. Tissue‡ (4-5 g) was ground for 15 minutes with an equal volume of sand and of chilled isotonic saline buffered with 1% of Sorensen's phosphates to pH 6.9. The mixture was diluted to 150 ml with cold saline, then centrifuged at 5° as shown in Table I.

Under the phase contrast microscope the

TABLE I.

Time (minutes)	Force (X gravity)	Sediment	Supernatant
3	74	Fraction 1	recentrifuged
15	74	" 2	" "
15	159	" 3	" "
15	283	" 4	" "
15	423	" 5	" "
15	636	" 6	" "
15	1130	" 7	" "
30	1130	" 8	Fraction 9

low speed fractions 2-4 appeared to contain large aggregates and amorphous clumps of both pigmented and non-pigmented materials. In fraction 5 a large majority of the particles were pigmented and in the one μ size range; a small number of aggregates of these granules were also present. In addition, some particles were found which were almost invisible in ordinary light but were demonstrable by phase contrast. The proportion of dark pigmented granules to these "grey" particles was approximately 80/20. Other, highly refractile granules variable in size and shape and ranging from 2 μ to 4 μ were occasionally seen. In fractions 6-9, unit granules comprised the major portion of the particulate material but the proportion of melanin granules to "grey" particles gradually decreased.

Fraction 5 was resuspended in 5 ml of cold distilled water and centrifuged for 15 minutes at 74x gravity. The supernatant fluid was then centrifuged for 15 minutes at 423x gravity. The sediment so obtained, resuspended in cold distilled water, proved to be the most homogeneous preparation of melanin granules obtained from the S-91 melanoma. Roughly 80% of all the elements present were shown by phase contrast to be pigmented. Under the electron microscope this fraction was found to consist principally of elliptical particles, the major axes of which ranged from 0.30 μ to 0.45 μ and the minor axes from

* For the last paper in this series, see Mason, H. S., *J. Biol. Chem.*, Jan., 1948, in press.

¹ Smith, D. T., *Bull. Johns Hopkins Hosp.*, 1925, **36**, 185.

² Herrmann and Boss, M. B., *J. Cell. and Comp. Physiol.*, 1945, **26**, 131.

³ Mason, H. S., *Ann. N. Y. Acad. Sci.*, in press.

⁴ Greenstein, J. P., Turner, F. C., and Jenrette, W. V., *J. Nat. Cancer Inst.*, 1940, **1**, 377.

† Loaned by the Army Air Forces, Wright Field.

‡ The tumor material was contributed by Dr. Glenn H. Algire.

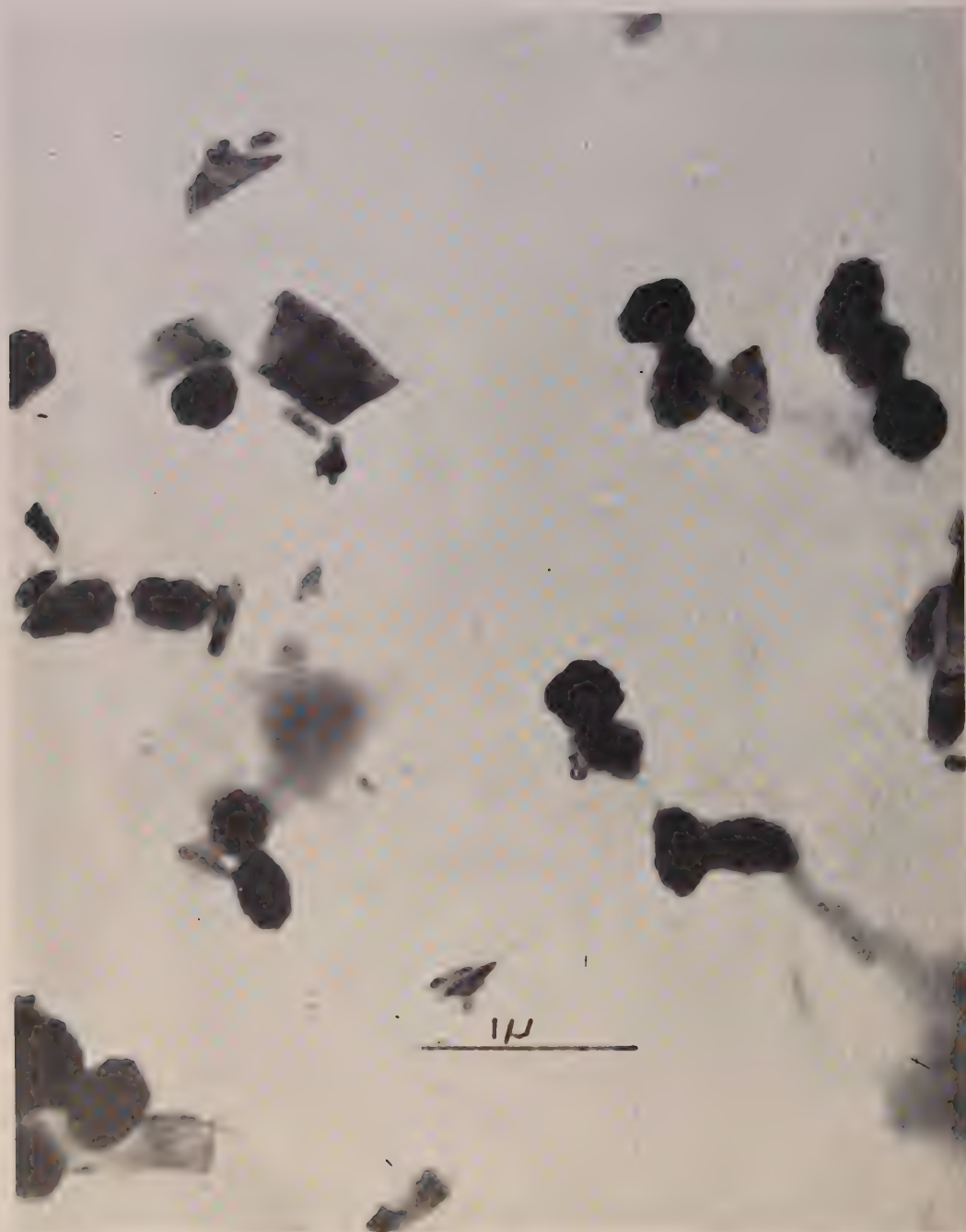


FIG. 1.
Melanin granules from the S-91 melanoma.

0.25 μ to 0.40 μ . The melanin granules themselves appeared almost opaque to the electron beam and no internal structure could be discerned (Fig. 1, 2).

Melanin granules from the Harding-Passey transplantable mouse melanoma. The process utilized above proved effective in concentrating the pigment granules from this source.[†]



FIG. 2.

Melanin granules from the S-91 melanoma, gold-shadowed at an angle of 1 to 5.

The fractions obtained corresponded in content to those from the S-91 melanoma, and under the electron microscope these melanin

granules appeared identical in size range and shape to those from the S-91 melanoma; no internal structure was revealed. (Fig. 3, 4).

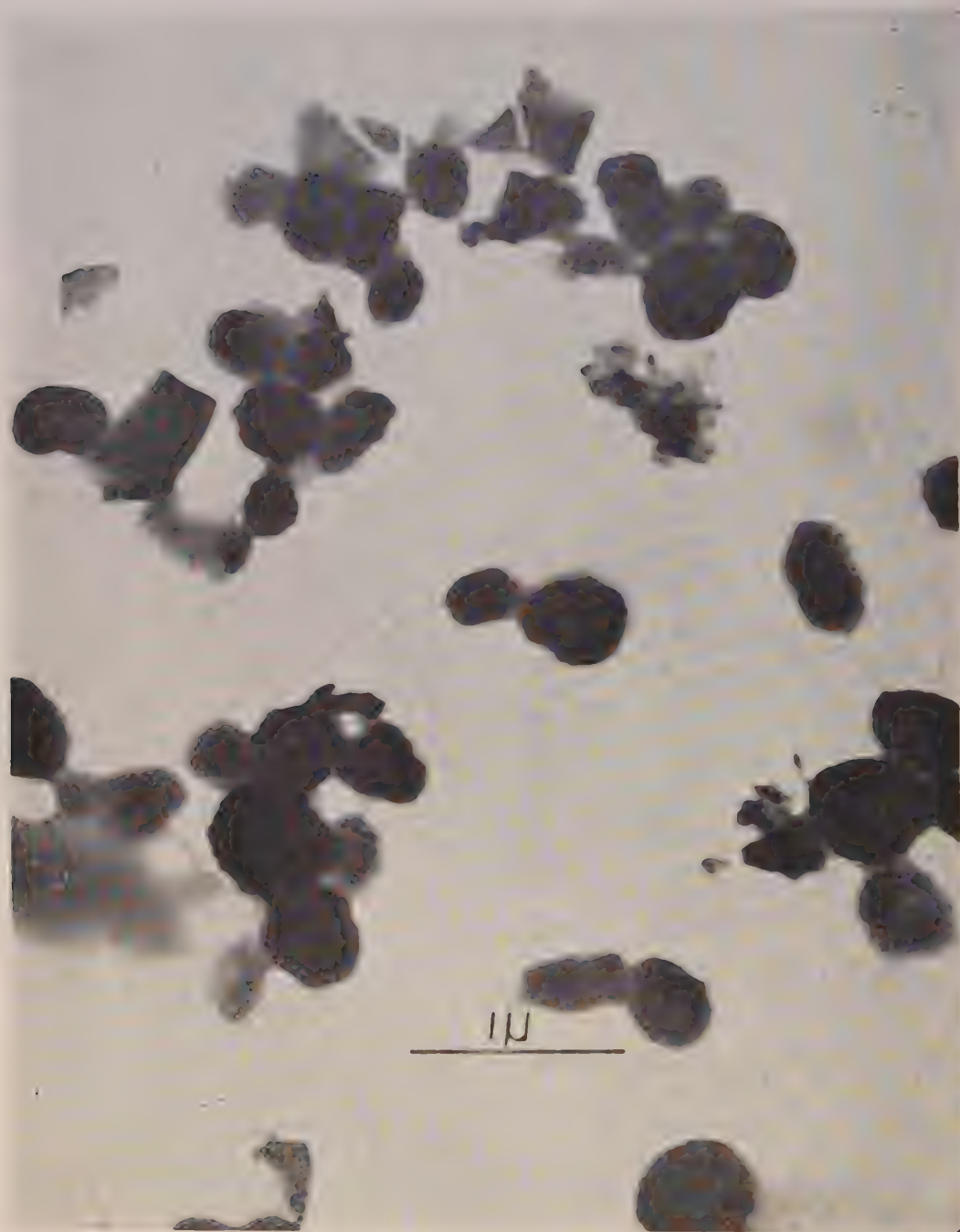


FIG. 3.
Melanin granules from the Harding-Passey melanoma.

Amelanotic melanoma. The specimens⁵

⁵ Algire, G. H., *J. Nat. Cancer Inst.*, 1944, 5, 151.

were prepared and centrifuged in the manner described for the two tissues above. In the phase contrast microscope no evidence of pigmented granules could be found in any frac-



FIG. 4.

Melanin granules from the Harding-Passey melanoma, gold-shadowed at an angle of 1 to 5.

tion but clumped material and unit particles ranging from $0.5\ \mu$ to $7\ \mu$ were observed. Under the electron microscope, few of these

particles corresponded in size or shape to melanin granules from the parent melanoma.

Melanin granules from beef iris, ciliary body



FIG. 5.
Melanin granules from the beef choroid.

and choroid. Ciliary body, iris and choroid were separated from twenty beef eyes.⁶ Each

⁶ Friedenwald, J. S., Herrmann, H., and Moses, R., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 421.

tissue was ground with sand and cold buffered

§ Beef eyes were obtained from freshly slaughtered animals through the courtesy of the Schluderberg-Kurdle Corp.



FIG. 6.

Melanin granules from the beef choroid, gold-shadowed at an angle of 1 to 5.

saline as before and the suspensions centrifuged at 74x gravity for 15 minutes; the pellets were resuspended in cold saline and re-centrifuged at the same speed. The combined

supernatant fluids were spun at 3672x gravity for 30 minutes, and the pellets in each case were resuspended in 10 ml of cold distilled water. The ciliary body preparation then

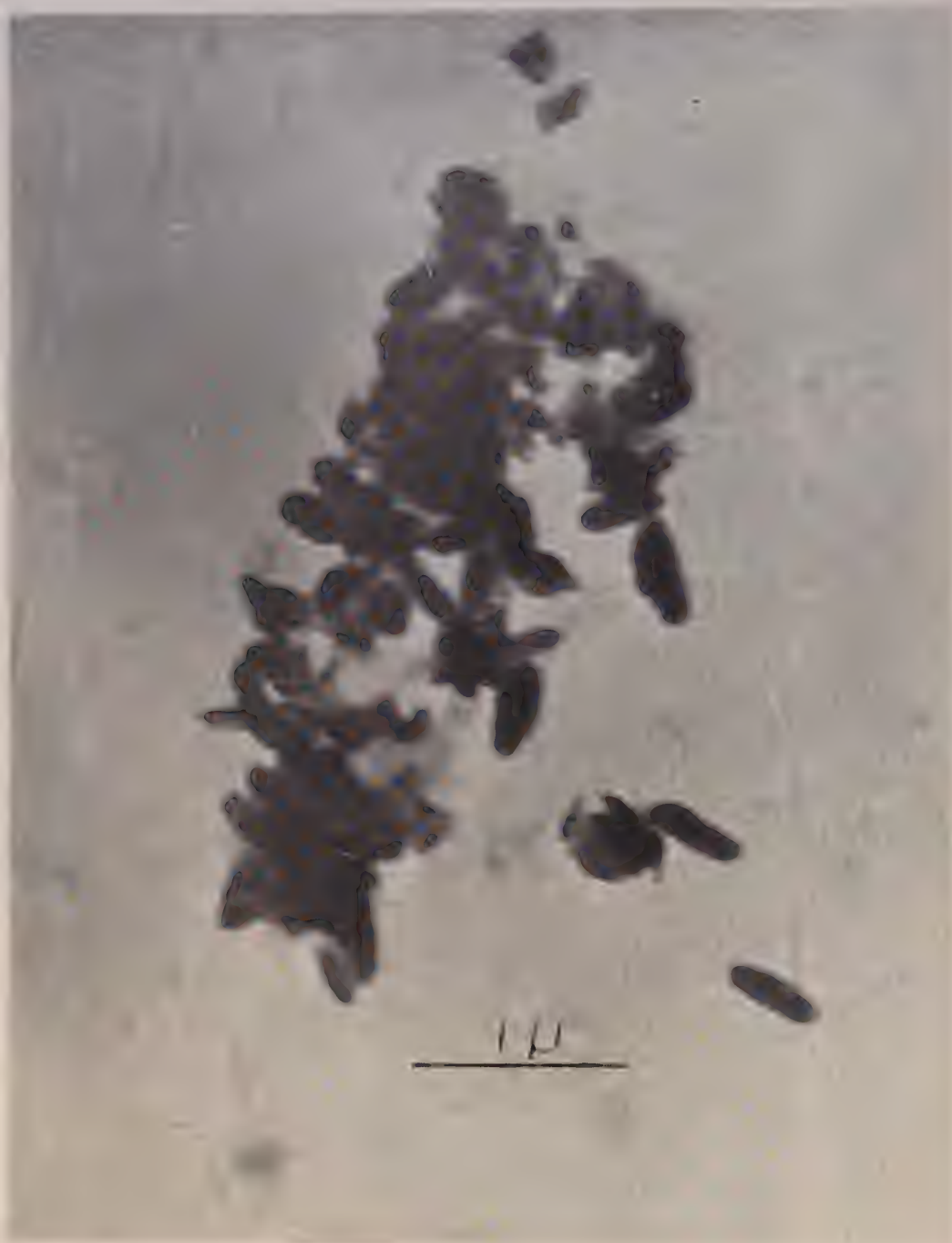


FIG. 7.
Melanin granules from human skin.

corresponded to the granule preparation of Herrmann and Boss.² Under the phase contrast microscope it was observed to consist of

approximately 95% melanin granules which varied widely in shape and size, together with extraneous material including colorless nuclei.



FIG. 8.
Melanin granules from human skin, gold-shadowed at an angle of 1 to 5.

The particles in each preparation were further sorted by removing aggregated material at 74x gravity (15 minutes), then deposit-

ing the major portion of the melanin at 423x gravity (15 minutes) and resuspending the pellet in 5 ml of distilled water. Under the

phase microscope the preparations appeared homogeneous with respect to melanin granules. In the electron microscope the granules varied from circular to elliptical shape, $0.30\ \mu$ to $2.00\ \mu$ across; they were sharply defined but again failed to reveal internal structure (Fig. 5, 6).

Melanin granules from human skin. A fresh specimen of colored human skin measuring 6 cm by 22 cm was obtained through the courtesy of Drs. John Wirth and Emerson Y. Gledhill. The heavily pigmented epidermis was readily scraped away from the dermis with a blunt scalpel and ground with 2 g of white sand and 5 ml of cold buffered saline for 15 minutes. The resultant slurry was diluted with 25 ml of chilled saline and centrifuged for 15 minutes at 74x gravity. The supernatant fluid was then centrifuged for 15 minutes at 423x gravity. All pigment was deposited in the pellet, which was resuspended in cold distilled water and cleared of aggregated material by centrifuging at 74x gravity for 15 minutes. Under the phase contrast microscope colorless globules about one μ in diameter were observed to constitute about 10% of the bodies visible. Of the pigmented particulates about 35% were rod-shaped and another 35% were small spheres of uniform shape and size. The remainder of the particulate material appeared as large clumps of dark granules. Under the electron microscope globular, rod-shaped, and clumped elements were observable (Fig. 7, 8). The rods varied in size from $0.10\ \mu \times 0.40\ \mu$ to $0.18\ \mu \times 0.60\ \mu$. The pigmented globules appeared to have diameters between $0.20\ \mu$ and $0.30\ \mu$. The photographic images were uniformly dense and no indication of internal structure could be discerned.

The melanin-containing pseudoglobulin from the S-91 melanoma. This material was prepared by following the directions of Greenstein, Turner, and Jenrette.⁴ No difficulty was encountered in repeating the procedure, but the pigmented pseudoglobulin could be deposited almost completely by centrifugation for 15 minutes at 970x gravity. Under the phase contrast microscope the preparation appeared to consist of pigmented particles,

much of which was held together in clumps by background material of low refractive index. Nuclei and an occasional whole cell were also seen. Under the electron microscope the pigmented particles proved to be granules of the same size range and shape as those obtained from the S-91 melanoma by the differential centrifugation procedure given above.

Discussion. Melanin granules are characterized with optical microscopes the resolving power of which—approximately $0.2\ \mu$ —is of the same order of magnitude as the granules themselves.⁷ The electron microscope has a resolving power of the order of $0.005\ \mu$ and is, therefore, the instrument of choice for investigation of granule form. However, it does not reveal the presence of pigment as such. We have therefore established the identity of the micrographed particles with melanin granules by optical means. This identity was, in addition, indicated by the relative absence of the typical pigmented forms in granule preparations from the amelanotic melanoma.

The regular and rounded shapes of a representative group of melanin granules now observed with the electron microscope establish these particles as formed elements. The alternative that they consist simply of precipitated aggregates of a metabolic end-product is obviated; melanin is evidently deposited according to a pattern. The demonstration that melanin granules unchanged in shape or size also make up the melanopseudoglobulin obtained from transplantable mouse melanomas shows that these granules may aggregate in the presence of ammonium sulfate and be redispersed in a measure upon dialytic removal of the salt.

Discontinuities in the micrographic images of melanin granules would give some indication of gross structure. The present series of micrographs however are uniformly dense across the images and conclusions with respect to internal structure cannot be drawn.

Summary. 1. Melanin granules were obtained by differential centrifugation from the S-91 and Harding-Passey transplantable mouse melanomas, from the choroid, ciliary

⁷ Russell, E., *Genetics*, 1946, **31**, 327.

body and iris of the beef eye, and from colored human skin. These preparations were characterized with the electron microscope.

2. The melanin-containing pseudoglobulin

from the S-91 melanoma was shown to consist of melanin granules unchanged in shape or size with respect to granules obtained by centrifugation.

16114

Evaluation of a New Test for the Effect of Vitamin P on Capillaries.*

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In our work with rutin¹ we felt the need for a quick test for vitamin P like effects on the capillaries. The method described by Majovski *et al.*² appeared to have the advantage of speed and simplicity necessary for the assay of a series of compounds.

The test proposed by Majovski *et al.*² consists of the following procedures: Control and test mice are placed in a small jar which is connected by means of a stop-cock and pressure tubing to a five-gallon bottle. The larger bottle is evacuated by means of a vacuum pump to 45 mm of mercury pressure. Upon opening the stop-cock, the pressure in the smaller jar containing the mice quickly drops to 70 mm of mercury pressure. After a 60-second exposure to this decreased pressure the mice are removed and observations are made on the extent of pulmonary hemorrhage. Control mice exhibited a greater degree of pulmonary hemorrhage than did those "protected" by prior administration of certain citrus peel fractions. Thus, it was assumed that this method affords a means of measuring changes in vascular fragility or permeability as influenced by various agents.

* Aided by a grant from the John D. and Fannie K. Herz Fund. The Department is in part supported by the Michael Reese Research Foundation. We are obliged to the Abbott Co. for a supply of rutin.

¹ Raiman, R. J., Later, E. R., and Necheles, H., *Science*, in press.

² Majovski, G. J., Lesser, A. J., Hanson, H. C., Carne, H. O., and Thienes, C. H., *J. Pharm. and Exp. Therap.*, 1944, **80**, 1.

In the report of this method, the authors made mention of control animals which failed to conform to the usual reaction and indicated that there seemed to be some correlation between the "presence or absence of food in the stomach and these peculiarities." This observation was repeated by Kibrick and Goldforb³ who reported "protection" afforded by starving the animals prior to their use in the test as compared with fed mice. Besides, Kibrick and Goldforb³ found that a number of preparations of the hesperidin group did not afford protection to the mice against pulmonary hemorrhage. They admit, however, that the drugs used may have had varying and unknown contents of vitamin P activity. We felt that the observations of Kibrick and Goldforb did not obviate the usefulness of this method as a test for vitamin P activity, as feeding and fasting of the mice were factors easily controlled.

A few preliminary trials with two groups of fed mice, one of which had received rutin, showed that the animals which had been fed only exhibited gross pulmonary hemorrhages, while those which had been fed and which also had received prior treatment with rutin, showed no pulmonary hemorrhages. Thus it appeared that control of the feeding-fasting factor retained the method's capacity for testing for vitamin P activity.

We have found, however, in the course of our work, that the method is not specific for

³ Kibrick, A. C., and Goldforb, A. F., *J. Pharm. and Exp. Therap.*, 1944, **82**, 211.

TABLE I.

Agent	Amt of drug intraperitoneally	Protection	No. of mice
Simultaneous controls of all tests	Untreated	No	48
Acetate buffer, pH = 7.75	0.15 cc	"	3
Rutin in acetate buffer, pH = 7.75	1 mg in 0.15 cc	Yes	4
Propylene glycol	0.15 cc	"	6
Rutin in propylene glycol	1 mg in 0.15 cc	"	12
Ethylene glycol	0.15 cc	"	4
Glycerol	" "	No	3
Ethyl alcohol	" "	Yes	4
Oxalic acid	2 mg in 0.15 cc	No	3
Hypertonic saline	0.15 cc	*	5
Glucose	2 mg in 0.15 cc	Yes	4

* Questionable.

vitamin P and are reporting our results here in order to save other workers time and labor.

Observations. On running control mice which received as prior treatment only the fluids used in dissolving the agents which we desired to test for their effects on vascular permeability and fragility, we found that some solvents by themselves afforded equal protection against the hemorrhages induced by exposure of the animals to decreased pressure as the specific agents believed to decrease vascular fragility and permeability. Therefore, we tried the effect of several other agents. Table I lists the agents tested and their ability to protect adult white mice of 20-25 g body weight and of both sexes against the pulmonary hemorrhages. No effect of sex was noted in the tests.

Our method deviated from that of Majovski *et al.* only in that the pressure to which the animals were subjected was slightly higher. We used a pressure of 85 mm instead of 70 mm of mercury.

No apparent pulmonary hemorrhages in treated mice as compared with grossly hemorrhagic lungs of untreated controls simultaneously exposed to the decreased pressure was called "protection," and similar degrees of hemorrhage in control and test animals was declared "no protection."

All agents were administered by intraperitoneal injection 35 minutes before exposure to the decreased pressure.

It might be mentioned that most of the "protected" animals survived significantly longer in the decreased pressure and after-

wards than did the controls, but the correlation between survival time and protection is not absolute.

Hypertonic saline afforded "questionable" protection because complete protection was never achieved, but in several trials, the mice given hypertonic saline exhibited significantly less hemorrhage than did the controls.

Except rutin, the agents which were found to protect the animals by this method are generally accepted to have no effect on vascular fragility or permeability. Most of the solvents used for rutin afforded as much protection as did rutin itself. Also glucose, which is an integral part of the molecule of both hesperidin and rutin, afforded protection against the pulmonary hemorrhages.

We conclude therefore, that this test is non-specific for the vascular effects of vitamin P.

Summary. The test proposed by Majovski *et al.* for evaluating the effects of vitamin P on capillaries was subjected to a critical study.

The experience of others that previous feeding affects the results of the test was confirmed, but this objection can be overcome by using starved mice only.

The present tests demonstrate that Majovski's method is non-specific, however, because a number of solvents used for the rutin, as well as other substances, gave mice the same protection against low atmospheric pressure as rutin did. The drugs which protected mice are not known to have any effect on capillaries; they were: propylene glycol, ethylene glycol, ethyl alcohol and glucose.

Effect on Cardiac Glycogen of Intravenously Administered Sodium Acetoacetate, Sodium Beta-Hydroxybutyrate and Sodium Butyrate.

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Following pancreatectomy^{1,2} or in alloxan diabetes,³ the glycogen content of the liver and skeletal muscles is rapidly depleted, but the glycogen content of the heart is increased. In some recent experiments,⁴ we have shown that in rats the amount of glycogen stored by the myocardium parallels changes in the blood ketone level induced by dietary means. In general, the glycogen stores of the liver and skeletal muscles tend to vary inversely with those of the heart. These observations suggested that ketosis may be a causative factor in increasing the deposition of cardiac glycogen, though decreased insulin availability may be important. With the idea of minimizing changes in insulin activity, experiments of shorter duration were carried out.

Method. Two groups of experiments were done using adult male white rats of the Sprague-Dawley strain. The animals of the first group (Table I) were fasted for 24 hours; those of the second group (Table II) were unfasted. Each group was divided into several series, one serving as a control series, the others being subjected to intravenous infusion of 0.9% sodium chloride, 3% sodium chloride, 10% sodium butyrate, 10% dl-beta-hydroxybutyrate or 10% sodium acetoacetate solution. The solutions were adjusted to a pH of 7.2; the volume infused varied from 5 to 7½ ml; the duration of the infusion was 4 hours; and the time of sacrifice was one hour after termination of the infusion. The animals were anaesthetized with sodium

pentobarbital and the trachea was cannulated routinely except in the uninfused control series of the first group, which was anaesthetized and sacrificed at the time the infusions were begun in the other series of the group.

Since the temperature regulating mechanism in the rat was greatly affected by the anesthetic, heat was applied in an attempt to keep the temperature of each individual animal near the normal level. At a room temperature below 30 to 31°C, supplementary heat from lamps was generally necessary.

At the time of the sacrifice, blood samples were obtained for glucose and ketone body determinations and samples of heart, liver and skeletal muscle were removed for glycogen determination. The various analyses were carried out by methods described earlier.³

Results and Discussion. Table I shows that in the fasted series receiving infusions of sodium dl-β-hydroxybutyrate and sodium butyrate, the cardiac glycogen stores were 654 ± 27 and 653 ± 34 mg % respectively, significantly above the values of 502 ± 26 and 510 ± 16 mg % found in the fasted series receiving respectively no treatment or an infusion of 0.9% sodium chloride solution.

The glycogen stores in the skeletal muscle were essentially the same in the experimental and the control series. The liver glycogen levels in the series receiving saline (532 ± 63 mg %) were significantly higher than in the untreated series (183 ± 32 mg %) or the series receiving sodium dl-β-hydroxybutyrate (112 ± 48 mg %). The very great standard error for values obtained for liver glycogen after sodium butyrate infusion makes it impossible to attach any significance to the data on liver glycogen in this series.

The only remarkable finding in blood ketone values was the low level of ketones in the series receiving the saline solution. In

¹ Cruickshank, E. W. H., *J. Physiol.*, 1913, **47**, 1.

² Fisher, N. F., and Lackey, R. W., *Am. J. Physiol.*, 1925, **72**, 43.

³ Lackey, Robert W., Bunde, Carl A., Gill, A. J., and Harris, Leroy C., *PROC. SOC. EXP. BIOL. AND MED.*, 1944, **57**, 191.

⁴ Lackey, Robert W., Bunde, Carl A., and Harris, Leroy C., *Am. J. Physiol.*, 1946, **145**, 470.

TABLE I.
Tissue Glycogen, Blood Ketones, and Blood Sugar in 24-Hour Fasted Rats Given Sodium *dl*- β -Hydroxybutyrate and Sodium Butyrate by Intravenous Infusion.

Solution infused	No. of animals	Glycogen* mg per 100 g tissue			Blood ketones mg per 100 ml†	Blood sugar mg per 100 ml
		Liver	Heart	Skeletal muscle		
None	17	183 $\pm$ 32†	502 $\pm$ 26†	396 $\pm$ 28†	17.6	115
Isotonic saline	12	532 $\pm$ 63	510 $\pm$ 16	385 $\pm$ 22	4.6	85
Sodium β -hydroxybutyrate	9	112 $\pm$ 48	654 $\pm$ 27	421 $\pm$ 15	54	111
Sodium butyrate	13	464 $\pm$ 264	653 $\pm$ 34	362 $\pm$ 54	15.8	139

* As glucose.

† Standard error.

‡ As hydroxybutyric acid.

TABLE II.
Tissue Glycogen, Blood Ketones, and Blood Sugar in Non-Fasted Rats Given Sodium *dl*- β -Hydroxybutyrate and Sodium Acetoacetate by Intravenous Infusion.

Nature of treatment or solution infused	No. of animals	Glycogen* mg per 100 g tissue			Blood ketones mg per 100 ml†	Blood sugar mg per 100 ml
		Liver	Heart	Skeletal muscle		
Untreated	10	1660 $\pm$ 200†	451 $\pm$ 34†	434 $\pm$ 15†	3.2	129
Dummy operation	16	2368 $\pm$ 276	510 $\pm$ 17	504 $\pm$ 24	3.4	116
Isotonic saline	11	3381 $\pm$ 309	615 $\pm$ 27	604 $\pm$ 16	7.6	134
Hypertonic saline	11	2537 $\pm$ 330	627 $\pm$ 27	551 $\pm$ 20	8.5	127
Sodium β -hydroxybutyrate	12	807 $\pm$ 251	681 $\pm$ 42	566 $\pm$ 37	22	136
Sodium acetoacetate	11	1051 $\pm$ 254	724 $\pm$ 45	614 $\pm$ 52	17.3	159

* As glucose.

† Standard error.

‡ As hydroxybutyric acid.

this the blood sugar level was also lower. Increases in the blood sugar level followed administration of sodium butyrate but not administration of β -hydroxybutyrate,

The rather large increase in cardiac glycogen in the series receiving sodium butyrate and sodium *dl*- β -hydroxybutyrate, without a concomitant increase in glycogen deposition in either the liver or the skeletal muscle, supports our previously stated concept of a relationship between ketonemia and increased glycogen in the myocardium. The increase in blood sugar level after the administration of sodium butyrate has been observed before^{4,5} and may be related to the increased cardiac glycogen; but observations that the elevation of the blood sugar level was not great and that the muscle glycogen stores were not increased suggest that the underlying factors which selectively increased the cardiac glycogen in this case were the same as those

in the ketosis of prolonged fasting, which is not accompanied by an increase in blood sugar level.⁴ In the fasted series in which *dl*- β -hydroxybutyrate was given, the increased cardiac glycogen was not associated with an increase in blood sugar.

In the fasted series infused with saline solution there was no change in the skeletal muscle, or cardiac glycogen. The increase observed in the liver glycogen may have been attributable to the decrease in muscular activity incident to anesthesia or to the fact that in fasting rats the liver glycogen stores are depleted in the early stages of the fast, but tend to be replenished as the fast is prolonged.⁶ The pronounced lowering of blood ketones and blood sugar in this series is difficult to explain, but may be related to the effect of sodium chloride on the insulin producing mechanism or the anti-insulin or anti-ketogenic mechanism, an action which

⁵ Markees, Silvio, *Klin. Wchnschr.*, 1941, **20**, 1260.

⁶ Bunde, Carl A., and Lackey, Robert W., unpublished data.

has been discussed by McQuarrie⁷ and Lewis, McKee, and Longwell.⁸

Table II records data from unfasted animals. The cardiac glycogen levels of 681 ± 42 mg % and 724 ± 45 mg % found in the experimental series receiving sodium β -hydroxybutyrate and sodium acetoacetate, respectively, were the highest observed. These series had also the lowest liver glycogen stores (807 ± 251 and 1051 ± 254 mg % respectively) and the highest blood ketone levels; the muscle glycogen levels (566 ± 37 and 614 ± 52 mg %) differed little from those found in the control series receiving the hypertonic and the isotonic saline solution (551 ± 20 mg % and 604 ± 16 mg % respectively). Thus the same parallelism is observed between the occurrence of ketonemia and increased cardiac glycogen stores in this as in the fasted series of animals.

Quite unexpectedly, the non-fasted animals receiving isotonic or hypertonic sodium chloride solution showed higher values for cardiac glycogen, skeletal muscle glycogen, blood ketones and blood sugar than did animals subjected only to dummy operations. These effects were contrary to those observed after similar saline infusion in fasted animals. The meaning of this difference is not apparent to us. The increase in blood sugar observed in the series receiving sodium acetoacetate raises the same question of inter-relationship with cardiac glycogen storage as that discussed in connection with the fasted series receiving sodium butyrate.

No attempt was made in these studies to control the factor of alkalosis which must have followed our administration of the sodium salts of acids capable of being metabolized. Deuel and coworkers⁹ have concluded

that alkalosis is inimical to hepatic glycogenesis and this may explain the lowering of liver glycogen in certain of our series. Long and Evans¹⁰ observed a lowered cardiac glycogen in rats with pronounced alkalosis, induced by giving them sodium bicarbonate. In our animals cardiac glycogen increased in spite of this adverse condition.

The data presented seem to give some additional support to the idea that there exists a parallelism between blood ketone levels and the deposition of glycogen in the myocardium. Since an artificially produced ketonemia may increase the glycogen stores in the heart within a few hours, either with or without a preliminary fast of short duration, the relationship between ketonemia and cardiac glycogen storage may well be a direct one rather than one indirectly attributable to the decreased insulin availability resulting from conditions giving rise to endogenous ketonemia.

Summary. In experiments carried out on adult male white rats under sodium pentobarbital anaesthesia, intravenous administration of ketone bodies resulted in an increase in the glycogen stores of the heart. Concomitant increase in the glycogen stores of the liver and skeletal muscle did not occur.

⁷ McQuarrie, Irvine, *Essays in Biology*, p. 413, University of California Press, Berkeley and Los Angeles, 1943.

⁸ Lewis, Robert C., Jr., McKee, Frances S., and Longwell, Bernard B., *J. Nutrition*, 1944, **27**, 11.

⁹ Deuel, Harry J., Jr., Butts, Joseph S., Blunden, Harry, Cutler, Charles H., and Knott, Leslie, *J. Biol. Chem.*, 1937, **117**, 119.

¹⁰ Long, C. N. H., and Evans, G. T., *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 186.

Temporal Relation Between Contraction of Right and Left Sides of the Normal Human Heart.

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Knowledge of the normal temporal relationship of contraction of the two auricles and ventricles is important in various clinical conditions. Only indirect or semi-direct evidence of the activities of the various cardiac chambers has been available so far in clinical cases. As a new method permits their direct observation, a study of normal subjects has been undertaken, as a preliminary to clinical studies.

Precedents. On the basis of simultaneously recorded right ventricular pressures, carotid surface pulses and ballistocardiographic recoils (direct method for the right ventricle only), Hamilton and co-workers¹ found an asynchronous ejection of the two ventricles in man. Either ventricle may precede the other.

The only available study on man with similar technique was briefly reported by Chamberlain and co-workers.² These authors studied the ascending aorta and the first part of the pulmonary artery by means of the electrokymograph. The study was made either with a single pickup device and subsequent tracings or with 2 separate pickups and 2 galvanometers. In the latter instance two simultaneous separate tracings were recorded, permitting comparison. These authors found a variable time relationship of the pulses of the 2 vessels; either ventricle might precede the other; in a few cases the pulses were simultaneous.

Method. The method used in this study is based on the combination of the fluoroscope, a photoelectric pickup unit, a screen with a slit and an electrocardiograph. The apparatus,

originally described by Henny, Boone and Chamberlain^{3,4} and called "electrokymograph" was subsequently modified by us together with Rappaport^{5,6} and the name "fluorocardiography" was proposed, as being more descriptive of the method.

The pickup unit is adjusted under fluoroscopic control so that the motion of a small section of the margin of the cardiac silhouette is seen within the slit. Any outward motion is recorded as a positive wave on the tracing; any inward motion, as a negative wave. Identification of these waves is possible by the simultaneous recording of a tracing of the heart sounds. The recorded tracings are similar to pulse tracings (arteries) and to volume tracings of the ventricles or auricles. Therefore, no new names are necessary for the waves, these being the same as in sphygmograms or cardiograms.

It is to be noted that the motions of the ventricular borders are somewhat less typical when recorded in the lateral positions even if the beginning and end of the various waves bears the normal temporal relation to the heart sounds.

Two Sanborn apparatus for fluorocardiography and a Sanborn Tri-beam Stethocardi-ette were used for the study. Observations were made on 8 normal subjects between the ages of 14 and 46.

The general method previously described by us for the positioning of the slit^{5,6,7} was followed. However, the simultaneous recording of

³ Henny, G. C., and Boone, B. R., *Am. J. Roentgen.*, 1945, **54**, 217.

⁴ Henny, G. C., Boone, B. R., and Chamberlain, W. E., *Am. J. Roentgen.*, 1947, **57**, 409.

⁵ Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *New England Heart Assn.*, Feb. 24, 1947.

⁶ Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, in press, 1948. Note I.

¹ Hamilton, W. F., Attyah, A. M., Remington, J. W., Wheeler, N. C., and Witham, A. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 266.

² Chamberlain, W. E., Boone, B. R., Ellinger, G. F., Henny, G. C., and Oppenheimer, M. J., *Fed. Proc.*, 1947, **6**, 88.

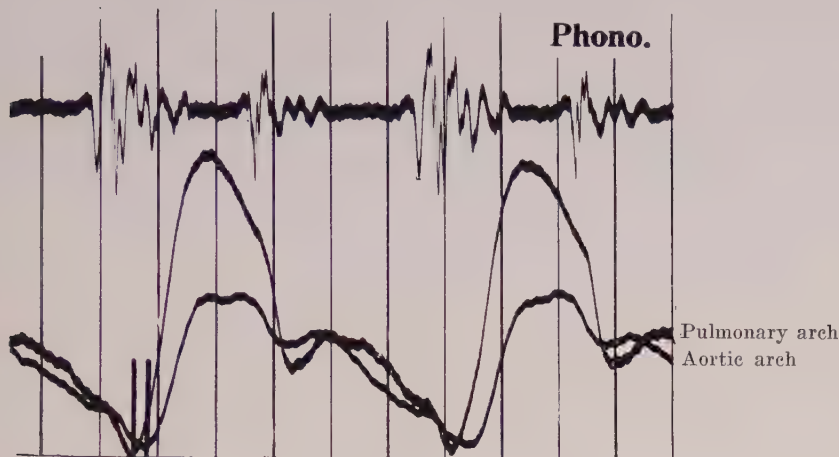


FIG. 1.

Simultaneous fluorocardiograms of the pulmonary and aortic arches. Phonocardiogram. The marks indicate the beginning of systolic expansion of the arteries.

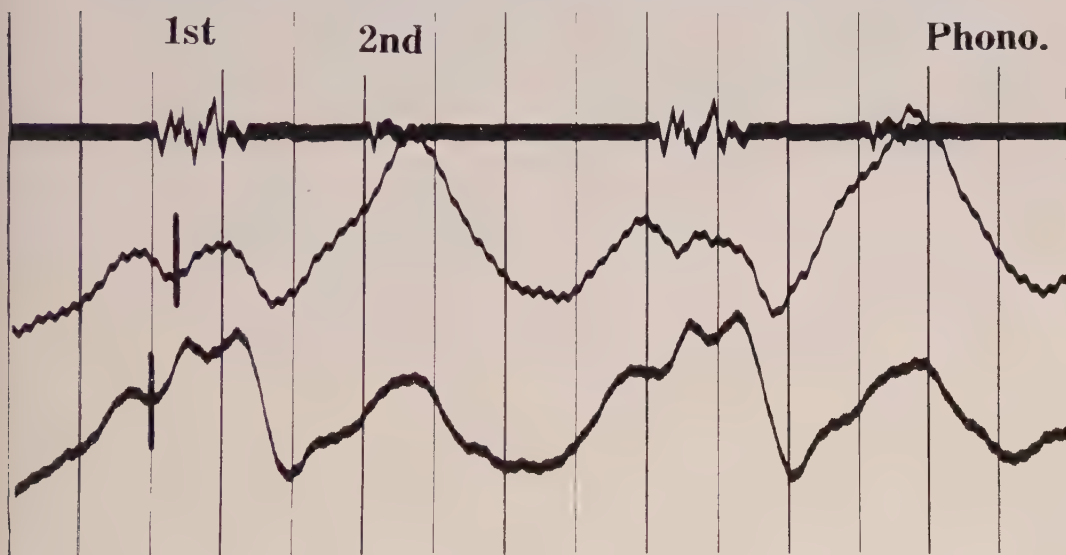


FIG. 2.

Simultaneous fluorocardiograms of the right (bottom) and left (middle) auricles. Phonocardiogram.

The marks indicate the maximum depth of the auricular contraction. The other waves bear a similar temporal relationship.

the activities of the right and left heart presented new problems, which were solved in the following way: (a) The study of the pulsations of the pulmonary arch and of the aortic arch was made with the subject in the postero-anterior position by applying the slits of the 2 pickup units across the borders of the curves corresponding to the 2 large ves-

sels. (b) The study of the pulsations of the 2 auricles was made with the subject in the right oblique position by applying the slits, one below the right bronchus (left auricle), the other much lower (right auricle). (c) The study of the pulsations of the 2 ventricles was made in the left lateral position applying one slit as low as possible across the anterior border of the cardiac shadow (right ventricle) and the other across the lower posterior

⁷ Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, in press, 1948. Note II.

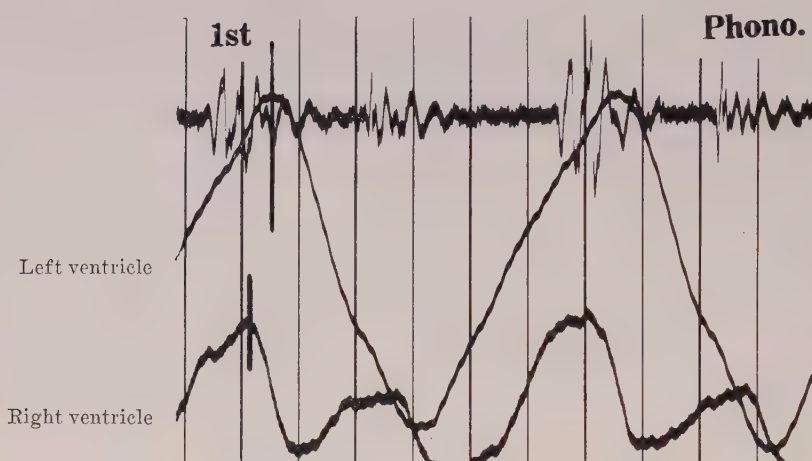


FIG. 3.

Simultaneous fluorocardiograms of the right and left ventricles. Phonocardiogram. The marks indicate the beginning of ventricular ejection.

border (left ventricle).

Results. (1) The simultaneous tracings of the pulsations of the pulmonary artery and the aortic arch (Fig. 1) show the following time relationship: (a) the rise of the pulse of the pulmonary artery precedes that of the aorta by 0.020 to 0.025 seconds (20 to 25 msec). (b) The peak of the pulse of the pulmonary artery precedes that of the aortic pulse by about 0.03 seconds (30 msec).

(2) The simultaneous tracings of the pulsations of the 2 auricles (Fig. 2) show the following time relationship: (a) The beginning of the auricular contractions is not always apparent owing to the rotation of the patient. When visible, the right auricle precedes the left. (b) The maximum depth of contraction is reached by the right auricle about 0.025 seconds (25 msec) before the left. (c) The maximum dilatation of the auricle, at the moment in which the auriculo-ventricular valve opens, is reached by the right auricle from 0.025 to 0.030 seconds (25 to 30 msec) before the left.

(3) The simultaneous tracings of the pulsations of the anterior and posterior aspects of the heart, namely of the 2 ventricles (Fig. 3) show that these bear the following relationship: (a) The beginning of ventricular contraction (isometric contraction) cannot be accurately ascertained in the lateral posi-

tion. (b) The beginning of the ejection period is indicated by a sharp fall of the tracing after the maximum has been reached subsequent to auricular contraction. This occurs about 0.025 seconds (25 msec.) earlier for the right ventricle (anterior surface) than for the left ventricle (posterior surface).

Temporal relation between contraction of right and left sides of the heart are further shown in the schemes of Fig. 4.

Comment. These observations concerning the temporal relations in cardiac action confirm each other. Actually, the final conclusions concerning the time relationship of the ventricular pulsations are based on the correlation of tracings obtained from the auricles indicating the opening of the A-V-valves; from the ventricles showing the beginning of ejection from both ventricles; and from the large vessels revealing the beginning rise of the aortic and pulmonic pulses. The evidence indicates that the right ventricle contracts first, and the interval between the contractions of the 2 ventricles is between 0.025 and 0.030 seconds (25 to 30 msec). The same interval exists between the pulmonic and aortic pulses. The latter is due to various facts: (a) the pulsation of the middle arch is actually that of the left branch of the pulmonary artery,⁸ taking place, therefore, somewhat after the beginning of ejection of

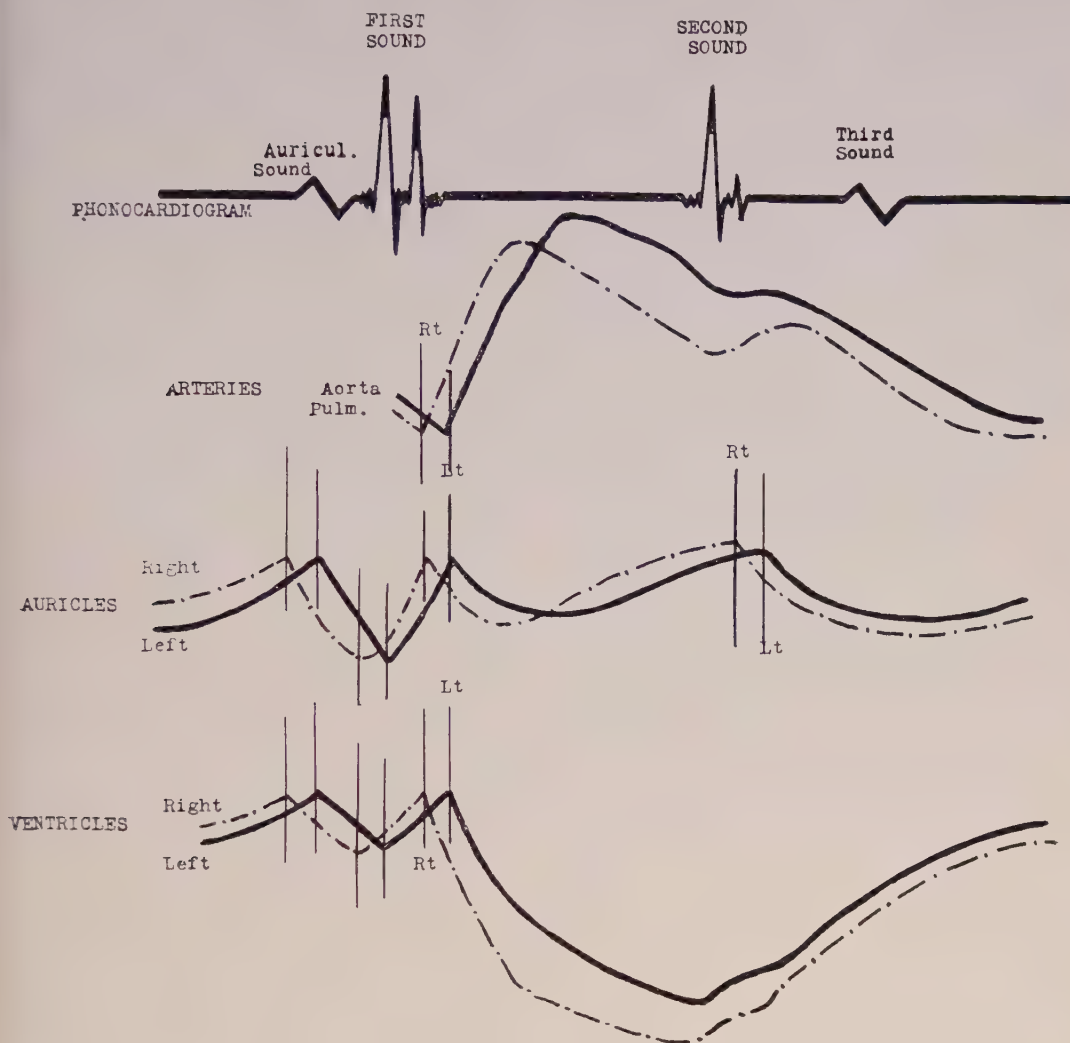


FIG. 4.

Temporal relation between contraction of right and left sides of the normal human heart. Schemes made from tracings of the various subjects.

the right ventricle. (b) The pulsation of the aortic arch takes place some time after the beginning of the ejection of the left ventricle. (c) The speed of the pulse wave is lower in the lesser than in the greater circulation, as indicated by Hamilton⁹ and confirmed by us.⁷ Therefore, in spite of the different length of the 2 large vessels, it is understandable that

the time relationship between their pulses turns out to be about the same as that between the beginning of ejection of the 2 ventricles. We can conclude that all data point to the fact that under normal conditions the right ventricular contraction precedes the left. This fact is in accord with the demonstration that, while the left side of the septum is excited before the right, the external wall of the left ventricle is reached after that of the right (Lewis¹⁰). The time

⁸ Chavez, L., Dorbecker, N., and Celis, A., *Am. Heart J.*, 1947, **33**, 560.

⁹ Hamilton, W. F., in *Howell's Textbook of Physiology* by John Fulton, Philadelphia, Saunders, 1946, p. 841.

¹⁰ Lewis, T., *The Mechanism and Graphic Registration of the Heart Beat*, London, Shaw, 1925.

interval between excitation of the right and left ventricles was found by Lewis to be only 0.01 seconds. As we found it, the interval was greater; this may be attributed to the fact that we measure the interval between the beginning of the ejection from the two chambers. We know that in the human heart the isometric contraction of the right ventricle lasts 0.01 sec. (Richards and co-workers¹¹) but we do not yet know the duration of the isometric contraction of the left. It is likely that the higher pressure existing in the aorta causes a slightly longer duration of the period of tension of the left ventricle, thereby exaggerating the delay already existing.

Our observations on the pulsations of the auricles indicate that, here too, there is a brief interval, the right auricle contracting before the left. Again our observation confirms known physiological facts. In the dog, the sino-auricular impulse reaches the left atrial appendage 0.015 seconds later than the right (Lewis,¹⁰ Wiggers¹²). It is logical

to assume that the interval is longer in the human heart owing to the greater length and difference in length of the muscular bundles leading from the sino-auricular node to either auricle respectively.

The constancy of our findings is in contrast with the variability of results of previous studies.^{1,2} This can be explained by the fact that those studies were performed partly with indirect measurements¹ or with a different technique.²

Summary. The temporal relation between the contractions of the left and right heart chambers was studied by means of fluorocardiography and simultaneous recording of 2 pulse tracings on one strip. The study was performed on 8 normal subjects and included the observation of the pulsations of the aorta and the pulmonary artery, both auricles and both ventricles. In all observations the contraction of the right auricle preceded that of the left auricle and the contraction of the right ventricle that of the left. The delay of action of the left chambers was found to be between 25 and 30 milliseconds.

¹¹ Richards, D. W., Cournand, A., Motley, H. L., Dresdale, D. T., and Ferrer, M. I., *Trans. Assn. Am. Phys.*, 1947, in press.

¹² Wiggers, C. J., *Physiology in Health and Disease*, Philadelphia, Lea and Febiger, 1944.

16117

The Effect of a *Sporosarcina ureae* Preparation on Tumor Cells *in vitro*.*

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The report that cell division in sea urchin eggs was inhibited by penicillin¹ was the incentive for these experiments designed to test the effect of microbial products on tumor

cells. Cornman² and Lewis³ found tumor cells in tissue culture damaged or killed by the addition of suitable concentrations of penicillin, though Lewis believed the selective carcinoclastic effect was due to impurities in the preparations.

In experiments not otherwise reported, we injected penicillin into Webster strain mice bearing spontaneous mammary carcinomata. Damage to the tumor in the form of hemorrhage, necrosis, and pyknosis was apparent but only after 50-100,000 units of penicillin

* We are indebted to Dr. Clyde K. Emery for a grant which made this work possible.

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¹ Henry, R. J., and Henry, M. D., *J. Gen. Physiol.*, 1945, **28**, 405.

² Cornman, I., *J. Gen. Physiol.*, 1944, **28**, 113.

³ Lewis, M. R., *Science*, 1944, **100**, 314.

were administered daily for more than a week.

A search for other carcinoclastic microbial products was then undertaken. For a survey of a large number of micro-organisms, *in vivo* test is too slow and interpretation is likely to be uncertain. Accordingly the test chosen was the histological condition of surviving tissue slices, as used in metabolism studies, after incubation for 24 hours with the bacterial preparation under investigation. A slice of the liver of the same animal was used as an indicator of the selectivity of the preparation for tumor or normal tissue. Penicillin, it was found, could be used to exclude extraneous micro-organisms.

Materials and Methods. Webster strain mice bearing spontaneous carcinomata, and pigmented rabbits carrying the transplantable Brown-Pearce carcinoma supplied the tumor and liver slices used in these experiments. Tissues which showed extensive necrosis or were otherwise unusual were discarded.

Mice were killed by a blow on the head and rabbits by injecting air into a marginal vein. The tumor and liver were removed, rinsed in physiological saline solution, and the tissues sliced into sections not more than 0.3 mm thick, either with a straight razor or a Stadie⁴ microtome. Mouse liver was generally cut by hand, while mouse tumor and rabbit liver and tumor were more conveniently cut on the microtome. The slices were rinsed through 3 baths of physiological saline solution before being placed in 20 ml beakers for incubation. Each beaker contained 4 ml of the medium, a slice of tumor, and as a control tissue, a slice of the autologous liver.

All procedures were carried out as aseptically as possible.

Previous tests showed that tissues incubated in serum under conditions later described preserved a more nearly normal gross and microscopic aspect than they did when incubated in balanced salt solution. Heterologous serum appeared to be as favorable as the homologous serum, consequently the basic medium consisted of rabbit serum ob-

tained from blood collected by aseptic cardiac puncture to which 100 mg of dextrose and 100,000 units of penicillin (Squibb) were added per 100 ml of serum before use. The usual procedure employed 3.5 ml of serum containing sugar and penicillin plus 0.5 ml of the culture or preparation being tested.

A shaker-incubator⁵ equipped to hold thirty 20 ml beakers allowed the agitation to be carried out at any desired speed, temperature, and under any preferred water-saturated gas mixture. Tissues in these experiments were incubated for 24 hours at 37°C under 95% oxygen and 5% carbon dioxide and shaken at 80 cycles per minute.

All preparations were run in duplicate, and with each set of experimental beakers a pair of beakers containing only serum and the tissue slices were incubated as control. In experiments not involving the use of penicillin in all beakers, an additional control pair without penicillin was run.

Specimens of tumor and liver were routinely fixed in Bouin's or Carnoy's fluid at the beginning of each experiment, and at the conclusion, all tissues were immediately fixed in preparation for microscopic examination.

The slices were sectioned at 8 μ in a plane parallel to the original slicing to give large area for examination. Orientation was assured by sandwiching the slices between sheets of agar gel prior to fixation.⁶ Hematoxylin-eosin was the routine stain.

The basic medium for growing the bacteria was as follows:

Yeast extract (Difco)	3 g
Peptone (Difco)	3 "
Dextrose	5 "
Tapwater	1 L

Adjusted to pH 8.0 with NaOH prior to autoclaving.

Most of the organisms tested grew well in this medium at room temperature. For the culture of *Sporosarcina ureae* and other urea decomposing bacteria 20 g urea was added to the above medium.

Results. In a general survey of micro-organisms whose products might selectively

⁴ Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, 1944, **154**, 687.

⁵ Dubnoff, J. W., in preparation.

⁶ Cohen, A. L., in preparation.

damage tumor tissue, the variety of growth habits made it necessary to modify the conditions of testing them. Thus bacteria which could grow rapidly in serum at 37°C were added as small inocula; those which might grow to some extent but not sufficiently to swamp the tissues were added either as loopfuls of rich culture to beakers containing 4 ml of serum minus penicillin or as 0.5 ml concentrates of rich cultures to 3.5 ml of serum containing penicillin; while those organisms which could not grow under the conditions described were added as 0.5 ml concentrates to each beaker containing 3.5 ml serum with penicillin added to suppress the growth of contaminants.

Some bacteria damaged both liver and tumor cells, the severity of injury ranging from slight eosinophilia and paling of the nuclei to complete disintegration. These were: *Pseudomonas caryocytanea*, *P. putida*, *P. sp.*, *Serratia marcescens*, *Escherichia coli communis*, *E. aurea*, *Shigella sonnei* and *Sarcina sp.*

The majority of micro-organisms tested exhibited no perceptible effect upon either the tumor or liver. In this category are *Pseudomonas indologens*, *P. ovalis*, *P. omnivorans*, *P. acidovorans*, *P. pyocyanea*, *P. tumefaciens*, *Vibrio metchnikovii*, *Rhodospirillum rubrum*, *Escherichia coli* (2 strains), *E. coli communis* (compare with *E. coli communis* of preceding list) *E. acidilactici*, *Aerobacter aerogenes*, *A. cloacae*, *Alcaligenes fecalis*, *Salmonella pullorum*, *Bacterium albolavus*, *Proteus vulgaris*, *Kurthia zopfii*, *Staph. citreus*, *Strep. anhemolyticus*, *Strep. sp.*, *Micrococcus ureae*, *M. cinnabareus*, *Sarcina sp.*, *Bacillus brevis*, *Aerobacillus polymyxa*, *Corynebacterium creatinovorans*, *Mycobacterium phlei*, *M. salmonicolor*, *M. sp.*, *Actinomyces coelicolor*, *Penicillium notatum*.

In addition to these organisms 3 trypanosomes, *Trypanosoma brasiliensis*, *T. lewisii*, and *Schizotrypanosoma cruzii* were tested because of Roskin's report⁷ of their use in producing tumor regression. They were ineffective in our test.

A perceptible specific carcinoclastic effect was produced by preparations of the following

organisms: Bacterium I (an unidentified, slender, motile, non-sporulating rod isolated from contaminated serum), *Spirillum virginianum*, *Phaemonas varians*, *Urobacillus pasteurianum*, and *Sporosarcina ureae*. Of these 5 organisms *Sporosarcina ureae* had the most pronounced differential destructive effect upon tumor tissues. It was selected, therefore, for further study.

After incubation with *Sporosarcina ureae* a slice of spontaneous carcinoma of the Webster mouse underwent the following degenerative changes: The nuclei became pyknotic, the cell membranes shrank and the characteristic acinar structure was largely obliterated. The liver slice cells appeared to be undamaged.

In the first experiments, *Sporosarcina ureae* was grown in 50 ml Erlenmeyer flasks containing 10 ml of culture medium at room temperature. The cultures were harvested at the peak of growth and 0.5 ml of culture was added to each beaker containing 3.5 ml of serum with mouse liver and tumor slices prepared as previously described. Experiments were made with and without the use of penicillin in the concentration of 1000 units per ml. No effect on the action of the *S. ureae* culture being observed, all further experiments were done with penicillin in the medium to safeguard tissues and serum against the growth of contaminants.

Because of the high concentration of ammonia in the *Sporosarcina ureae* cultures addition of 0.5 ml to 3.5 ml of serum gave a pH of 9.5. It was possible, then, that the specific toxicity of the *Sporosarcina ureae* culture to tumor cells resided in either the ammonia present in high concentration or the high pH induced rather than in a more specific toxic substance. To test these possibilities tumor and liver slices were incubated in the serum medium to which either ammonium hydroxide or sodium hydroxide were added to give a pH of 9.5 or higher. No damaging effect of either ammonium or hydroxyl ion at these concentrations was observed on either the tumor or liver cells. Nor did removal of the ammonia from various *Sporosarcina ureae* preparations lessen their

⁷ Roskin, G., *Cancer Research*, 1946, **6**, 363.

selective carcinoclastic effects.

In an attempt to enhance the effect *Sporosarcina ureae* was grown under the following different conditions:

A. Two-liter flasks containing 500 ml of culture fluid inoculated with *S. ureae* were incubated at 30°C for 48 hours with constant agitation. These heavy cultures harvested at the peak of growth produced a slighter effect than those grown under less favorable cultural conditions. Further experiments indicated that the carcinoclastic effect was greater with cultures which had passed the peak of growth.

B. A 500 ml culture was prepared, agitated at 30°C for 48 hours, and allowed to remain undisturbed at room temperature (ca. 22°C). After 48 hours a sample (BI) was lyophilized, and the remainder (BII) was lyophilized after 120 hours at room temperature. When reconstituted with distilled water sample BI showed little or no carcinoclastic effect, while tumor tissue exposed to sample BII was totally destroyed, the nuclei densely pyknotic, cell boundaries extinguished, and the acinar structure collapsed. There was no perceptible effect upon the liver.

In studies with whole culture, washed bacteria and cell-free medium, it was regularly observed that most pronounced carcinoclasia was obtained either with the washed concentrated cells or with whole old culture.

C. Plates were made of the yeast extract urea medium solidified with a 2% agar, heavily seeded with *S. ureae* and incubated at 30°C for 48 hours. The bacteria were washed off with distilled water, the washings collected and lyophilized. The bacteria from four 200 mm diameter petri dishes were resuspended in 20 ml distilled water after lyophilization, a few drops of methylene blue added as redox indicator, and the suspension divided into four 5 ml lots incubated 18 hours under the following conditions:

C I. Anaerobic at 55°C Methylene blue incompletely reduced.

C II. Anaerobic at 36°C Methylene blue incompletely reduced.

C III. Room temperature under toluene. Methylene blue incompletely reduced.

C IV. 30°C under toluene. Methylene blue

reduced in one-half hour. Toluene removed from C III and C IV by vacuum drying, residue reconstituted to original volume.

Preparation C IV, that autolysed under toluene at 30°C, produced complete disintegration of the tumor tissue and did not damage the liver as far as could be seen from stained preparations. Next in order of effectiveness were preparations C I and C II. C III had little effect.

Rabbit Brown-Pearce carcinoma was tested in a similar manner as the Webster strain mice carcinomata (above) and similar results were obtained.

The factor in *Sporosarcina ureae* toxic to tumor and not to liver cells appears to be contained within the bacteria and increases in the medium as these are aged. The evidence for this conclusion is the greater effectiveness of old cultures, the complete and selective destruction of tumor tissue by the bacteria autolyzed at 30°, and the ineffectiveness of the cell-free culture medium in which the bacteria had grown.

Discussion. As far as we are aware, the test method used in this study has not been employed hitherto in the study of or search for substances selectively toxic to tumor cells. It holds out the following advantages: it is rapid, economical, the one animal, even the one tissue, supplies control and experimental specimens, and indirect (immunological and vascular) effects of the test substances are inoperative.

Kidd⁸ used a somewhat analogous approach. He found that certain tumor cells after treatment with culture filtrates of *Aspergillus fumigatus* failed to grow after implantation, although there was no visible injury in the tumor cells.

Summary. The incubation of mouse spontaneous carcinoma, rabbit Brown-Pearce carcinoma, and their autologous liver slices with bacterial cultures showed in general that cells of both liver and tumor were uninjured or injured equally. Cultures of *Sporosarcina ureae* damaged tumor cells and left liver cells histologically unaffected. Evidence was obtained that the selective carcinoclastic factor

⁸ Kidd, J. G., *Science*, 1947, **105**, 511.

of *S. ureae* cultures was contained within the cells and released on autolysis.

We are indebted to Dr. C. B. van Niel of the Hopkins Marine Station for free access to the

culture collection, to Colonel Max Levine for cultures of several of the Enterobacteriaceae, and to Dr. William Negherbon for cultures of trypanosomes and detailed directions for their maintenance.

16118

Liver Injury in the Dog Following Use of 2-3-Dithiopropinol.* (BAL).

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Fifteen adult dogs furnish the basis for the observations which are to follow. The animals were kept in metabolism cages and given a diet of Purina Dog Chow. No restriction as to the amount was made in the diet or in the water intake. The dithiol was given intramuscularly at 9 a. m. and 9 p. m. on 4 successive days. The dogs were divided into 3 groups. The animals of Group I were given 5 mg per kilo of the dithiol per dose, those of Group II, 15 mg and the members of Group III, 30 mg per kilo. Prior to the commencement of the injections liver function studies were undertaken by the use of brom-sulfalein according to the technique devised by Rosenthal and White.¹ For the normal animals and those of Group I, such observations have been satisfactory in that all of the dye was removed from the plasma within half an hour. In the animals of Group II and Group III receiving respectively 15 and 30 mg of dithiol per kilo the rate of removal was invariably prolonged beyond the half-hour period. The percentage removal however during this and subsequent periods was extremely variable. The evidence for the development of a liver injury from the dithiol is not confined to such variable functional observations but has been ascertained by obtaining biopsy material from the livers and by the study of such tissue in those animals that came to autopsy. The tissue was stained for lipid

material with Scharlach R and also with hematoxylin and eosin. A study of such material permits the following conclusions:

1. In the animals of Group I which received 5 mg of 2-3-dithiopropinol per kilo it was difficult if not impossible to ascertain by such a micro-chemical method whether or not there was any actual increase in lipid material in the hepatic epithelium over that which can be frequently observed in such tissue designated as normal. In such animals lipid material appears in the liver in the form of dust-like and larger particles or as minute droplets. This material is more noticeable in the periphery of the lobules following the use of a dithiol.

2. In the animals of Groups II and III which received either 15 or 30 mg of the dithiol per kilo, there develops a marked increase in stainable lipid in the liver lobules in the form of larger or smaller droplets which may obscure the nuclei of such cells and replace the greater portion of the cell cytoplasm. Such a development is more uniform and more marked in the periphery than in the central portions of the lobules. In general as this area of the lobule is reached the lipid material is not only reduced in amount but makes its appearance as small discrete droplets. This order of hepatic cell injury is more severe and develops earlier in the animals of Group III that were given 30 mg of the dithiol per kilo. In one animal of this group there developed by the fifth day of the experiment an extensive liver necrosis. The animal became comatose with

* 2,3-dithiopropanol (BAL), 10%, benzyl benzoate 20% in peanut oil.

¹ Rosenthal, S. M., and White, E. C., *J. A. M. A.*, 1925, **84**, 1112.

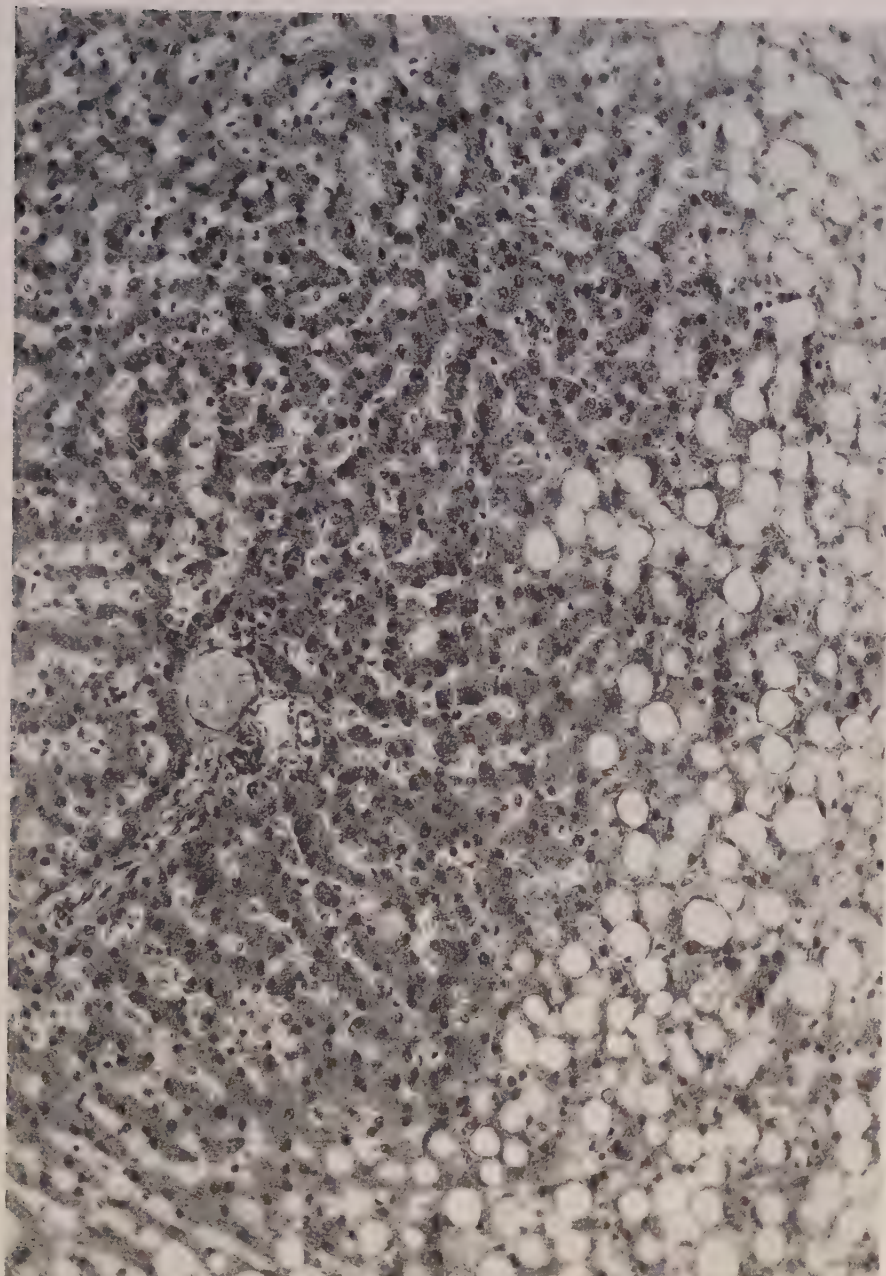


FIG. 1.

Microphotograph, Zeiss $\times 235$, hematoxylin and eosin. The figure is from biopsy material of the liver of animal 3, Group III, on the fourth day of the use of 2,3-dithiopropanol, 30 mg per kilo. Fatty changes indicated by the appearance of larger and smaller droplets of such material are more marked in the periphery of the liver lobule and diminishes as the area of the central vein is approached. In the periphery of the lobule cellular degeneration is marked while in the region of the central vein cell structure is well preserved.

an air-hunger type of respiration and died on the fifth day.

3. The experiments indicate the ability of a dithiol when given to normal dogs in an appropriately large amount per kilo to induce an hepatic injury which is characterized by fatty degeneration of the hepatic epithelium and rarely by a necrosis of this tissue. Such

observations do not necessarily imply that a similar order of cellular injury would be induced in the dog under the influence of an intoxication by salts of certain of the heavy metals. Such bodies offer a bond of union for the dithiols which in turn may prevent a toxic effect which they are capable of inducing in tissues of normal constitution.

16119

Influence of Diet upon the Hypertension and Nephrosclerosis Produced by Desoxycorticosterone Acetate Overdosage.*

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We have reported in a previous paper¹ that the hypertension and nephrosclerosis produced by administration of large amounts of anterior pituitary preparations may be prevented by decreasing the protein content of the diet from the normal of about 25% to 15%. In that publication attention was also called to the fact that adrenal enlargement was always marked in the hypertensive animals, which had been kept on a high protein diet, but was much less pronounced in the normotensive ones, which were given a low protein ration.

In order to clarify further the mechanism by which dietary protein affects the production of hormonal hypertension, we have now investigated its role in the production of the hypertension and nephrosclerosis induced by desoxycorticosterone acetate overdosage.

Material and Methods. *Production of hypertension.* Hooded castrated male rats, weighing 45 to 65 g, were unilaterally ne-

phrectomized and implanted subcutaneously with two 40 mg pellets of desoxycorticosterone acetate (DCA). The animals were then divided into 2 groups: one received a 30% (Diet I), the other a 15% (Diet II) casein ration, both containing a large amount (4%) of NaCl, (for details see Table I). At the end of 40 days the surviving animals were killed and their kidneys and adrenals weighed and sectioned for histologic study, after fixation in "Susa" mixture. Eight animals in Group 1 and 5 rats in Group 2 were killed on the 20th day.

Blood pressure determinations. The blood pressure was determined by carotid cannulation (care being taken to avoid hemorrhages) on a representative number of rats on the 20th day of the experiment and in all surviving animals the day before autopsy, excepting 3 animals in which we missed the determinations. In order to spare the animals no control determinations were performed at the beginning of the experimental period; this appeared permissible as we had previously established¹ that the normal blood pressure of such rats, under our conditions, is 108 ± 14 (standard deviation) mm Hg. Statistical considerations revealed that 108 ± 28 mm Hg would include 95% of all normal rats, hence 135 mm Hg was taken as the highest normal blood pressure. The direct method

* This work was supported by a grant from the Commonwealth Fund.

[†] Canada-Brazil Trust Fund Fellow, from the Laboratory of Endocrinology, Instituto Butantan, São Paulo, Brazil.

[‡] Canadian National Research Council Fellow.

¹ Dontigny, P., Hay, E. C., Prado, J. L., and Selye, H., in press.

TABLE I.
Composition of Two Basic Synthetic Diets.
(Parts per cent.)

Diets	I	II
Casein	30	15
Corn starch	59	74
Fat	1	1
Cod liver oil	1	1
Bulk	1	1
Sodium chloride	4	4
Mineral mixture	4	4
Supplements:		
Thiamine chloride	0.8 mg/100 g diet	
Riboflavin	" "	
Pyridoxine	" "	
Ca pantothenate	4.0 "	
Nicotinic acid	1.0 "	
Choline chloride	100 "	
Tocopherol acetate	10 mg/rat/weekly	

of blood pressure determination gives the mean pressure only.

Kidney lesions. The incidence and severity of the kidney lesions were estimated by a procedure previously described.³

Diets. The complete composition of the diets employed is given in Table I.

Experiments. In a first series of experiments each group consisted of 15 rats. The experiment was then repeated with 10 animals in each group. The results were almost identical and will therefore be described conjointly.

Since under certain conditions¹ choline chloride appeared to counteract the hypertensive action of anterior pituitary preparation, an additional group of 10 rats was treated exactly as those on Diet I but they received 10 times the amount of choline chloride indicated in Table I.

Results. Table II summarizes our results. The average hypertension given in Table II refers to the average of the hypertensive animals only. It seemed appropriate to us not to include in the calculation the few normotensive rats, in order to obtain the true hypertensive average.

Confirming previous findings of this laboratory² it may be seen in Group 2 that the implantation of DCA produced a marked elevation of the blood pressure. This hyper-

tension, which was present in almost all animals, was completely established on the 20th day of the experiment; it persisted for a period of at least 20 days which elapsed between the two blood pressure determinations. The kidney lesions in this group were also very prominent, as judged by the high incidence and severity of the pathologic changes.

We wish to emphasize particularly that a 15% casein diet does not prevent the production of hypertension and nephrosclerosis under these conditions. In fact our results show that at the end of 40 days 100% of the animals in Group 1 were hypertensive, with an average blood pressure of 161 mm Hg and had as marked kidney lesions as in the 30% casein group.

Ten times the usual amount of choline chloride (Group 3) effected no detectable improvement.

Discussion. Our earlier experiments¹ showed that the hypertension produced by anterior pituitary overdosage is prevented by a 15% casein diet; the present observations indicate that DCA-hypertension, on the other hand, cannot be prevented in this manner. According to the working hypothesis prevalent in this laboratory, the anterior pituitary increases the blood pressure by stimulating the adrenal cortex.^{4,5} Since it now appears that the DCA-hypertension is obtained even on low protein diets, it is probable that the site of action of dietary proteins is between the pituitary and adrenal cortex; either proteins increase the efficacy of the corticotrophic hormone or they stimulate corticotrophin production. Experiments on hypophysectomized rats are now under way in this Institute to elucidate this last-mentioned point.

Choline chloride had no influence upon the establishment of hypertension by DCA overdosage.

Summary. While the hypertension and nephrosclerosis produced in the rat by anterior pituitary preparations are prevented by low-

² Selye, H., and Pentz, E. I., *Canad. M. A. J.*, 1943, **49**, 264.

³ Hay, E. C., and Seguin, P., *Am. J. Physiol.*, 1946, **147**, 299.

⁴ Selye, H., and Stone, H., *J. Urology*, 1946, **56**, 399.

⁵ Hall, C. E., Dontigny, P., Beland, E., and Selye, H., *Endocrinology*, 1946, **38**, 296.

TABLE II.
Effect of Dietary Protein Concentration upon Hypertension and Nephrosclerosis Produced by Desoxycorticosterone Acetate Overdosage.
(Averages and standard errors.)

Groups	No. rats at end of exper.	Diets*	Final body wt	Nephrosclerosis		Hypertensive mean pressure		
				Incid. %	Severity %	Avg hypert.†		% hypert. rats. 40 days %
						20 days	40 days	
1	16	15% casein	130 ± 5.2	100	79	154 ± 2.7 (19)	161 ± 3.8 (15)	100
2	13	30% "	135 ± 7.0	100	79	160 ± 3.0 (25)	157 ± 5.9 (11)	82
3	8	30% " + 10 X choline chl.	144 ± 7.3	100	79	—	163 ± 9.9 (8)	87

* Diets I and II.

† Figures in parentheses refer to number of determinations.

protein diets, the otherwise similar lesions induced by desoxycorticosterone overdosage are largely independent of the dietary protein intake.

We are grateful to the Schering Corporation of New Jersey for desoxycorticosterone acetate and to Misses L. Derome and T. Hansen for technical assistance.

16120

Effects of Dithiobiuret on the Central Nervous System.*†

SOL ALTSCHUL. (Introduced by W. S. McCulloch.)

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Illinois Neuropsychiatric Institute, Chicago.

Astwood *et al.*¹ reported that 0.002% solution of dithiobiuret, substituted for drinking water, paralyzed and killed rats in 7 days although each took less than .5 mg per diem. Peripheral nerve, myoneural junction and muscle were normal in function. Muscle, peripheral nerve, brain, spinal cord, root and ganglion were microscopically normal. On return to drinking water, the animals promptly recovered, but while paralyzed, strychnine failed to induce convulsions. Effects of di-

thiobiuret on cortex and convulsant threshold is reported here.

Method. Dithiobiuret, prepared by the American Cyanamid Co., is sparingly soluble in water and as it goes into solution smells of hydrogen sulfide. Saturated solutions in saline or distilled water were used for local applications, intramuscular and intravenous injection, and 0.001 and 0.002% in tap water orally and intraperitoneally.

Two dogs, 3 cats and a fourth that had received 5 cc saturated solution intramuscularly for 8 days were used to determine effects of dithiobiuret when applied to cortex and injected intramuscularly or intravenously in animals under Dial.[‡] Electroencephalograms of the fourth cat and one rat that had received 0.002% dithiobiuret for 9 days were

* The author is indebted to Dr. R. O. Roblin of the American Cyanamid Co., for placing dithiobiuret at his disposal.

† The work presented in this paper was done in partial fulfillment of the requirements for the degree of Master of Science in the Graduate School of the University of Illinois.

¹ Astwood, E. B., Hughes, A. M., Lubin, M., Vanderlaa, W. P., and Adams, R. D., *Science*, 1945, **102**, 196.

‡ The author wishes to thank Ciba Pharmaceutical Products who kindly supplied this drug.

TABLE I.

Rat	Dosage and route, g/100 cc	Toxic change seen in rate	Threshold* prior to drug, volts	Threshold* prior to toxic changes volts	Threshold* during toxic changes, volts
1	.002 oral	severe	27.9†	50†	70-75†
4	" "	" (died)	45	50-55	70
5	" "	slight	45	50	55
7	" "	severe (pregnant)	40-45	55	70-75
14	" "	" (died)	—	—	—
15	" "	" "	—	—	—
16	" "	slight "	—	—	—
20	" "	died before results obtained	—	—	—
21	" "	severe	—	—	—
22	" "	" "	—	—	—
8	.002 I.P.	slight (died after inj.)	—	—	—
9	" "	severe	—	—	90
10	" "	" "	—	—	65
2	.001 oral	not toxic	40	50	—
3	" "	severe	46	45	65
6	" "	slight	45	50	75
17	" "	not toxic	—	—	—
18	" "	" "	—	—	—
19	" "	" "	—	—	—
11	.001 I.P.	severe	—	—	80-85
12	" "	" "	—	—	70
13	" "	" "	—	—	80

* Cortical threshold to electrically induced convulsions.

† A copper electrode was placed into the mouth; in all other rats a brass electrode was used.

I.P. Intraperitoneal.

taken to determine effects of prolonged administration. Normal animals were used as controls. Electrical activity of cortex was recorded by a 6-channel Grass electroencephalograph.

Rats were used to determine effects of dithiobiuret upon cortical threshold to electrically induced convulsions. Convulsions were produced by a Goodwin, condenser discharge, stimulator[§] with one electrode placed into the mouth; the other on the vertex of the scalp. Voltage was varied from 25 to 90 volts in 5 volt increments, while frequency of stimulation was 120 per sec. and time duration of the falling phase of each stimulus was 0.5 millise. Duration of stimulation in all cases was 5 sec. and the interval between attempts to induce convulsions was 20 to 30 min. Cortical threshold to electrically induced convulsions was defined as voltage (other factors remaining constant) required to produce toni-clonic activity lasting more than 5 sec. after stimulation. Sixteen rats received unrestricted amounts of dithiobiuret

orally and 10 rats received daily 10 cc doses of dithiobiuret intraperitoneally. Four rats given 0.002 and 3 rats 0.001% dithiobiuret orally were used to determine cortical threshold before, during, and after administration of the drug. Two rats receiving 0.002 and 3 rats 0.001% dithiobiuret intraperitoneally were used to determine cortical threshold only after toxic changes were seen. (Table I). After toxic changes in the animals were noted, 10% glucose in 10 cc doses were injected intraperitoneally. One dose was given to each of 6 rats and 2 doses were given to each of 2 rats.

Results. Electroencephalographic tracings of 4 cats and 2 dogs did not change after local application of dithiobiuret or after single intravenous and intramuscular injections of saturated solutions of dithiobiuret in doses ranging up to 100 cc. Prolonged administration of dithiobiuret in one cat and one rat failed to produce any significant difference in electrical activity of cortex from that of normal animals.

The following progressive physical changes were noted in rats 5 to 12 days after the

§ The Goodwin Stimulator used in this study was obtained through the courtesy of Lab-Tronics, Inc.

initial dose of dithiobiuret. First was a decreased ability to use their hind limbs, as indicated by a shuffling gait; the animals would lie still most of the time, unless their tails were pinched. Next, they completely lost the ability to use their hind limbs. Finally, the rats could not move at all and died. After toxic changes were first seen, it took from 2 to 7 days for severe changes and death to occur. A slight toxic reaction was defined as the beginning disturbance of gait, while a severe reaction was defined as an inability to use the hind limbs. Of 10 rats receiving 0.002% orally, 7 showed severe reactions, 2 showed slight reactions while one died before changes were noted. Five rats receiving daily intraperitoneal doses of 10 cc of either 0.001 or 0.002% solutions showed the same severe changes, which appeared however, a few days earlier. A sixth rat on 0.002% intraperitoneally died shortly after an injection, but had already displayed slight effects to previous doses. Of 6 rats on 0.001% orally, only 2 showed toxic effects.

With respect to the cortical threshold^{||} to electrically induced convulsions, the following results were noted. Prior to administration of dithiobiuret, 6 rats displayed typical toni-clonic muscular contractions of a grand mal type after stimulation at approximately 45 volts. A seventh rat had a cortical threshold of 28 volts. A copper electrode, however, was placed into its mouth while in all others a brass electrode was used. In these 7 rats stimulated after the drug had been given and before toxic changes were seen, a change in cortical threshold varying from a decrease of one volt to an increase in 20 volts was noted (Table I). The average change was a 6-volt rise in threshold. These same animals when showing toxic effects had a rise in the cortical threshold of 10 to 45 volts, with an average rise of 26 volts. The rat showing only 10 volts change died before severe toxic effects were present. In 5 animals, tested only

after toxic changes had been observed, the increase in cortical threshold ranged from 20 to 45 volts, with an average increase of 28 volts over the average cortical threshold of animals tested prior to administration of the drug. The form of the convulsions changed when the animals became intoxicated. Non-intoxicated rats responded to stimulation with typical toni-clonic seizures, while seizures in intoxicated rats were decreased in intensity with some animals showing sustained tonic seizures followed by minimal clonic movements. In these animals the voltage was raised until a grand mal type reaction occurred.

Recovery was not hastened by one or two daily intraperitoneal injections of 10 cc of 10% glucose.

Discussion. The effects of dithiobiuret in raising the cortical threshold to electrically induced convulsions is great only when the rats are almost paralyzed.

Since no change in electrical activity of cortex was detected in 4 cats, 2 dogs, and one rat after administration of dithiobiuret, the action can not be based on a disturbance in the electro-chemical system of the cortex responsible for the electroencephalogram.

Astwood *et al.* demonstrated that strychnine failed to induce convulsions in animals paralyzed by dithiobiuret. This, plus findings of ascending spinal paralysis in animals able to respond to painful stimuli, seems to indicate that dithiobiuret produces a functional change in the efferent system of spinal cord.

Summary. The effects of dithiobiuret on electrical activity of cortex were tested upon 4 cats, 2 dogs, and one rat. Normal animals or the same animals were used as controls. Twenty-two rats were used in chronic administration and 12 of these were used to determine cortical threshold to electrically induced convulsions. The significant findings were: (1) a lack of detectable electroencephalographic change in cortical activity of animals tested after immediate or prolonged administration of dithiobiuret, (2) cortical threshold to electrically induced convulsions of rats was raised by an average of approximately 26 volts and (3) the form of con-

^{||} The term "cortical threshold" is used in the accepted manner, but since a non-blocking amplifier was not available for recording local changes, cortical discharges that were blocked at a lower level, might have occurred prior to observed seizures.

vulsion in intoxicated rats was altered.

Conclusion. Dithiobiuret is an effective anti-convulsant in rats only when they are intoxicated. The site of action of dithiobiuret

is within the central nervous system; the most probable site is the efferent system of the spinal cord.

16121 P

Specificity of Eczematous Hypersensitivity to P-Aminobenzoic Acid Butyl Ester ("Butesin").

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The present study was carried out on a 15-year-old boy who was originally seen for a contact dermatitis resulting from the application of butesin picrate ointment to a burn on the wrist. Routine patch test with the commercial ointment, which contains 1% butesin picrate, yielded a strongly positive reaction with marked reddening, edema and vesiculation.

Experimental and results. Patch tests were carried out in this patient with a series of related compounds in order to establish the range of specificity of this reaction. The materials studied were dissolved in triethanolamine in 2½% concentrations. The patches were removed after 48 hours and the reactions observed for another 48 hours.

Patch tests with a series of homologous p-aminobenzoic acid alkyl esters including methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and amyl esters yielded positive reactions. Para-aminobenzoic acid and the alcohols which constitute the side chains of the esters yielded negative results. No reaction was obtained with the methyl ester of meta aminobenzoic acid.

Six commercial anesthetics which are derivatives of p-aminobenzoic acid with a tertiary amine in the side chain were tested with negative results. This series included procaine, larocaine, tutocaine, monocaine, butyn and pontocaine.

Eight local anesthetics, benzoic acid esters with a tertiary amine in the side chain but without an aromatic amino group, were tested.

TABLE I.
Effective Threshold Concentrations of p-aminobenzoic Acid Alkyl Esters.

Ester	Effective threshold concentrations
Methyl	1 : 10 ²
Ethyl ("benzocaine")	1 : 10 ⁶
Propyl	1 : 10 ⁸
Butyl ("butesin")	1 : 10 ⁶
Amyl	1 : 10 ⁶
Isopropyl	1 : 10 ⁶
Isobutyl	1 : 10 ³

These were alypin, metycaine, stovaine, nupercaine, diothane, phenacaine, apothesine and intracaine. All gave negative reactions.

The 7 substances which elicited positive reactions were then tested quantitatively to determine threshold concentrations at which a just perceptible reaction occurs. (Table I)

The table shows that the sensitivity to the methyl ester was relatively low. The sensitivity increased with lengthening of the side chain to reach a maximum with the propyl ester. Further lengthening of the side chains resulted in a decreased sensitivity. The iso compounds caused in both cases considerably less reaction than the isomer n-compounds.

Discussion. There are only a few studies dealing with multiple epidermal sensitivity. In one group of the studied cases, contact dermatitis can be provoked in a rather haphazard fashion by a number of not closely related compounds.¹⁻⁴ In the other group, however, the sensitivity has a rather narrow

range within a well defined chemical configuration.^{1,4,5,6} Such sensitivity patterns have been perfectly identical in different persons and therefore may be regarded as essential in the mechanism of the sensitization. In the group of procaine sensitive patients, for instance, 3 cases have been reported in which the sensitivity was restricted to those p-aminobenzoic acid derivatives which contain a tertiary amino nitrogen in the side chain.^{4,6} The case herein described is similar to 3 cases reported by Schwarzschild¹ in which the sensitivity pattern was restricted to the alkyl esters of p-aminobenzoic acid. Schwarzschild reported in one case that the methyl

ester of p-aminobenzoic acid caused considerably less reaction than the ethyl, propyl and isobutyl esters.

A repeatedly encountered feature of these group sensitivities is that maximum sensitivity is found not with the actually sensitizing compound but with one of the related homologues.^{4,6} This was the case in the present observation. The patient was sensitized to the butyl ester but was 100 times more sensitive to the propyl ester with which he apparently never had contact before. The sensitivity patterns studied so far in eczematous reaction can be well explained by Pauling's theory of antibody formation.⁷

Summary. A case is reported with eczematous hypersensitivity restricted to alkyl esters of p-aminobenzoic acid. No reaction was obtained with substances deviating from this basic structure in the ring or in the side chain.

⁷ Pauling, L., Campbell, D. H., and Pressman, D., *Physiol. Rev.*, 1943, **23**, 203.

¹ Schwarzschild, L., *Arch. Dermat. und Syph.*, 1928, **156**, 432.

² James, B. M., *J. A. M. A.*, 1931, **97**, 440.

³ Goodman, M. H., *J. Invest. Dermat.*, 1939, **2**, 53.

⁴ Strauss, M. J., *J. Invest. Dermat.*, 1947, **8**, 403.

⁵ Rostenberg, A., and Kanof, N. M., *J. Invest. Dermat.*, 1945, **6**, 201.

⁶ Rothman, S., Orland, F. J., and Flesch, P., *J. Invest. Dermat.*, 1945, **6**, 191.

16122

Mydriatic Activity of Some New Synthetic Anticholinergic Esters.*

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Numerous publications during recent years have indicated that mydriasis and cycloplegia may be brought about by drugs that are primarily anticholinergic in action. Nyman¹ determined the relative mydriatic activity in man of various alkaloidal and synthetic substances. Scopolamine, 1-hyoscyamine and atropine methyl-nitrate exceeded

the activity of atropine whereas synthetic agents such as trasentine, euphthalmine and di-n-butylcarbaminoylecholine sulfate (dibutoline) are distinctly less active. Fromherz² found that the diphenylglycolic acid ester of γ -diethylamino- β,β -dimethylpropanol would produce maximum mydriasis in rabbits when instilled into the conjunctival sac in a concentration of 0.2%. Swan and White³ have described the mydriatic activity of dibutoline

* The authors wish to acknowledge the assistance of Miss Bernice L. Dertinger in the preparation of these data for publication.

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¹ Nyman, Ebbe, *Acta Physiol. Scand.*, 1942, **3**, suppl., 10.

² Fromherz, K., *Arch. f. exp. Path. u. Pharmacol.*, 1933, **173**, 86.

³ Swan, K. C., and White, N. G., *J. Pharm. Exp. Therap.*, 1944, **80**, 285; *Arch. Ophth.*, 1944, **31**, 289; *Am. S. Ophth.*, 1944, **27**, 933.

TABLE I.
 Spasmolytic Action of Anticholinergic Drugs.

Drug	Structure	Spasmolytic action against acetylcholine induced contractures (Isolated segments of rabbit ileum)
612	β -Diethylaminoethyl di- α -thienylacetate HCl	3 M*
1565	β -Diethylaminoethyl di- α -thienylglycolate HCl	50 M
606	β -Diethylaminoethyl phenyl- α -thienylacetate HCl	4 M
600	β -Diethylaminoethyl phenyl- α -thienylglycolate HCl	60 M
1597	β -Diethylaminoethyl cyclohexyl- α -thienylacetate HCl	17 M
623	β -Diethylaminoethyl cyclohexyl- α -thienylglycolate HCl	100 M
Trasentine	β -Diethylaminoethyl diphenylacetate HCl	1 M
109	β -Diethylaminoethyl diphenylglycolate HCl	33 M
Atropine sulfate		50 M

* Dilution of the drug in parts per million.

and recommend a concentration of 7.5% for clinical use. Ing, Dawes and Wajda⁴ determined the mydriatic activity in mice of a large number of new synthetic compounds and found several to be more potent than atropine. However, since these drugs were given intraperitoneally, direct comparison cannot be made with results obtained in other investigations of mydriatic substances wherein the material was applied externally to the eye.

Lands, Nash and Hooper⁵ described the pharmacology of a series of anticholinergic esters containing a few drugs with marked mydriatic effects. The synthesis of additional mydriatic drugs has permitted this laboratory to carry out further experiments in an effort to develop synthetic substitutes for atropine and homatropine. A portion of the results obtained is described in this communication.

Results. All drugs were screened for anticholinergic activity by determining the dilution required to abolish acetylcholine contractures in isolated intestinal segments of the rabbit (Magnus), as previously described.⁵ Results obtained are shown in Table I. Esters of acetic acid, disubstituted by phenyl, cyclohexyl, or α -thienyl groups, or combinations thereof, were tested for anticholinergic activity. Disubstitution in the acetate portion by unlike groups, such as phenyl/ α -thienyl (No. 606) or cyclohexyl/ α -thienyl (No.

1597), in the group of compounds tested, gave compounds which were more spasmolytic than either the corresponding diphenyl (trasentine) or di- α -thienyl analogues. The esters of disubstituted hydroxyacetic acid tested were from 6 to 33 times as spasmolytic (anticholinergic) as the nonhydroxy compounds. The most active compound, β -diethylaminoethyl cyclohexyl- α -thienylglycolate HCl (No. 623) exceeded atropine sulfate in spasmolytic potency.

Mydriatic action was determined in albino rabbits selected at random from our colony. The drugs, dissolved in distilled water, were instilled directly into the conjunctival sac; the eyes were held closed for one minute and upon release the excess drug was allowed to drain away. The pupillary response to strong direct illumination (100-watt electric light bulb with reflector held near the eye) was determined before and at intervals after drug instillation. The results obtained are shown in Table II. In general, mydriatic potency parallels spasmolytic potency. The disubstituted acetates, trasentine, No. 612, No. 606 and No. 1597 produce incomplete mydriasis when used at a concentration of 0.5 to 2.0%. The corresponding glycolates are effective mydriatics. No. 623 produces definite mydriasis in rabbits at a concentration of 0.02%. A 0.1% solution produces maximum mydriasis lasting more than an hour with some mydriasis lasting more than 7 but less than 24 hours. With a 1.0% solution there was prolonged mydriasis, corneal anesthesia and moderate irritation of the conjunctival

⁴ Ing, H. R., Dawes, G. S., and Wajda, Isabelle, *J. Pharm. Exp. Therap.*, 1945, **85**, 85.

⁵ Lands, A. M., Nash, V. L., and Hooper, K. Z., *J. Pharm. Exp. Therap.*, 1946, **86**, 129.

membranes. None of the synthetic drugs described here is more mydriatic than atropine sulfate.

Because of limited solubility of the hydrochloride, other salts were prepared and tested. Results obtained are shown in Table III. The nitrate is completely soluble in water and equals the hydrochloride in its mydriatic potency. A 1.0% aqueous solution of this salt is slightly irritant to the conjunctiva but causes no demonstrable damage to either the cornea or conjunctiva. Solutions at a concentration of 2.0 and 5.0%, made isosmotic to tears by adding glycerine, were instilled into rabbit eyes daily for 5 days. At the end of the test period the eye was bathed with sodium fluorescein and the cornea examined for damage. Irritation was slight with the 2.0% solution and there were only a few fluorescent areas on the corneal surfaces. These were small, being about 1-3 mm in diameter. They disappeared rapidly after treatment was discontinued. The 5.0% solution, similarly applied, caused edema and hyperemia of the conjunctiva and nictitating membrane and by the 5th day of treatment there was a large amount of mucoid discharge and cloudiness in the corneas of the treated eyes. This cloudy area covered half of the corneal surface from the inferior corneal margin upward and was strongly fluorescent when bathed with sodium fluorescein. Recovery was rapid after medication was discontinued and by the 4th day no evidence of corneal damage or irritation could be found.

Gilman *et al.*⁶ have described significant local anesthetic effects for β -diethylaminoethyl diphenylglycolate HCl and related structures. The local anesthetic effect of a few of the drugs in our series has been determined in albino rabbits. The drugs were dissolved in distilled water to make a 1.0% solution of the hydrochloride salts and instilled directly into the conjunctival sac of the rabbit eye, as described above for the determination of mydriatic action. The medial surface of the cornea was touched lightly

with a blunted pencil at 2- to 5-minute intervals after drug application. The absence of the wink reflex was taken as an indication of anesthesia. The results obtained were: No. 606—20 min.; No. 600—18 min.; No. 109—5 to 20 min.; No. 623—43 min. Gilman *et al.*⁶ have reported that No. 109 and trasentine, in a 2.0% solution, cause corneal anesthesia for 18 and 21 minutes. No. 606 and 600 appear to have comparable anesthetic effects. No. 623 is distinctly more anesthetic than the above drugs.

Discussion. Lands *et al.*⁵ have shown that β -diethylaminoethyl acetate has a cholinergic action on the isolated intestinal segment. Anticholinergic action was observed in those compounds containing an hydroxyl and phenyl, cyclohexyl or α -thienyl groups on the acetate portion of the ester. High spasmolytic potency against acetylcholine induced contractures was associated with high mydriatic potency. Both actions appear to be the result of "blocking" at the cholinergic receptors of the effector cells. Direct comparison of relative effects on these two types of cells is difficult because of the differences in methods used for studying and evaluating the actions described here. The determination of mydriasis was by direct instillation of drugs into the conjunctival sac. The actual concentration of drug in the aqueous humor would be influenced greatly by the rate at which it passed across the corneal barrier and by the rate of absorption from the aqueous humor. On the other hand, the isolated intestinal segments were bathed directly by a known concentration of drug. However, it should be noted that drugs with low potency for the intestinal segment were also poor mydriatics (No. 612, 606 and trasentine).

Blicke and Kaplan⁷ have reported results obtained by Dr. John G. Beall with a large group of mydriatic drugs synthesized by them. They report that a 2.0% solution of the γ -dimethylamino- β , β -dimethylpropanol esters of mandelic, β -phenyl- α -hydroxypropionic, β -phenyl- β -hydroxypropionic and β , β -diphenyl- β -hydroxypropionic acids are with-

⁶ Gilman, A., Goodman, L., Thomas, J. M., Hahn, G. A., and Prutting, J. M., *J. Pharm. Exp. Therap.*, 1942, **74**, 290.

⁷ Blicke, F. F., and Kaplan, H. M., *J. Am. Chem. Soc.*, 1943, **65**, 1967.

out mydriatic action whereas the esters of the corresponding tropic and benzoic acids are strongly mydriatic. The importance of the structure of the acid portion of the molecule is similarly emphasized in our series. The mydriatic and spasmolytic potencies of the β -diethylaminoethyl glycolates described here were much greater than those found for the corresponding acetates. Although two of the drugs described by us (Nos. 600 and 623) have spasmolytic action exceeding that of atropine, none is as strongly mydriatic as atropine. The reasons for this discrepancy are not readily apparent.

Summary. The β -diethylaminoethyl esters of di- α -thienyl-, diphenyl-, phenyl- α -thienyl- and cyclohexyl- α -thienylglycolic acids are anticholinergic drugs with significant mydriatic action. Of these, the cyclohexyl- α -thienylglycolic acid ester (No. 623) is the most mydriatic substance. The nitrate salt of this ester is very soluble in water and causes only small damage to sensitive tissues when used daily for several days at a concentration of 1.0 or 2.0%. It is suggested that this substance may have clinical applications as a mydriatic and cycloplegic agent.

16123

Enzyme Studies on Human Blood. I. Proteolytic Activity Associated with a Fraction of Plasma.*

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The demonstration of the proteolytic enzyme activity of the blood serum and plasma has attracted the attention of many workers since Dastre¹ originally inferred such an activity in serum. Delezenne and Pozerski² reported the proteolytic activity of serum which had been shaken with chloroform. Tagnon^{3,4,5} studying this problem more thoroughly found that this enzyme is an euglobulin. Tillett and Garner⁶ extracted from cultures of hemolytic

streptococci a substance capable of dissolving fibrin clots. Milstone⁷ later showed that this material did not act on highly purified fibrinogen and that a plasma euglobulin fraction was necessary for the "fibrinolysis." Christensen^{8,10} and Christensen and MacLeod⁹ found that the substance isolated from the streptococcic culture acted as an activator ("streptokinase") transforming the inactive plasma enzyme ("plasminogen") into "plasmin." They believe that this "plasmin" differs from trypsin but is identical with the chloroform-activated enzyme. Ferguson *et al.*¹¹ employing "fibrinogenolysis" as an assay technic of

* The protein fractions and the dried whole plasma were obtained through the courtesy of Dr. E. J. Cohn. They were prepared in collaboration between the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., and the Division of Biologic Laboratories of the Massachusetts Department of Public Health from blood collected in collaboration with the American Red Cross.

¹ Dastre, 1893, cited by Tagnon,³ 1942.

² Delezenne, C., and Pozerski, E., *C. R. Soc. biol.*, 1903, **55**, 690.

³ Tagnon, H. J., *Science*, 1942, **95**, 334.

⁴ Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 525.

⁵ Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

⁶ Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

⁷ Milstone, H., *J. Immunol.*, 1941, **42**, 109.

⁸ Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

⁹ Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

¹⁰ Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.

¹¹ Ferguson, J. H., Travis, B. L., and Gerheim, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 285.

"tryptic" activity demonstrated such after "streptokinase" activation in Fraction I of human plasma.

Much less conclusive data are available for the demonstration of the active enzyme without previous treatment with "streptokinase," chloroform, or other agents. Schmitz¹²⁻¹⁴ isolated an enzyme by adsorption on calcium-fibrin and subsequent fractionation. It produced a slight increase in non-protein nitrogen when added to casein or gelatin. He postulated the existence in plasma of a kinase-trypsin-trypsin inhibitor system. However, recent evidence shows certain dissimilarities between trypsin and the chloroform—or the "streptokinase" activated-enzyme.^{9,15} Taylor *et al.*¹⁶ made observations on the proteolytic activity of plasma protein fractions prepared by low temperature-ethanol separation¹⁷ and without previous chloroform treatment. Qualitatively, the enzyme activity was tested by observing the lytic action of the fractions on a fibrin clot. "Plus" proteolysis is reported in one out of 4 whole plasma samples, 2 out of 6 Fraction I, and in both of Fraction III-2 studied. Edsall¹⁸ states that in Richert's method Fraction III-2 (or III-2,3) is treated with thrombin and the clot which forms adsorbs "plasminogen." This clot lysed spontaneously and was designated Fraction III-3. In 2 or 3 weeks at 0°C a slowly progressive proteolytic activity was demonstrated without the addition of any activator. Glazko¹⁹ also reports the proteolytic activity of Fraction III-3. Christensen¹⁰ observed that fractions of serum containing "plasminogen" will activate spontaneously in the absence of chloroform or "streptokinase"

in the refrigerator after several weeks, when the inhibitor content is low.

This report concerns experiments on human whole plasma and its primary protein fractions separated only by low temperature-ethanol procedure and the demonstration of considerable proteolytic activity in Fraction I without previous treatment with activators.

Methods and Material. The dried whole plasma and its fractions were stored in a domestic-type mechanical refrigerator. No special precautions as to temperature, humidity and air exposure were taken during weighing of samples. Unless otherwise specified, a test mixture consisted of equal volumes of 2% aqueous solutions of specified protein fractions and 2% suspension of "C.P." casein in 1/15 M Sorensen phosphate buffer of specified pH, both prepared on the day of incubation. With each test 2 controls were run simultaneously consisting of equal volumes of buffered casein and distilled water and equal volumes of protein solution and buffer. Each test and control mixture was overlaid with C.P. toluene, 1% of the total volume. As additional bacteriological control measure, frequent smears and cultures were taken. The pH was determined with a glass electrode electrometer. Incubation occurred at 37.5°C and the reactions were terminated by the addition of cold 20% trichloroacetic acid equal in volume to the reaction mixture. After one hour in the refrigerator, the precipitated proteins were separated by filtration. Either acid soluble total N or "tyrosine" or both were determined in duplicate on one cc aliquots of the filtrate. The micro-Kjeldahl-Nesslerization and the Folin-Ciocalteu²⁰ procedures, as employed in this communication, have average approximate sensitivities of 0.004 mg N and 0.001 mg tyrosine respectively per 1% on the transmission scale of the Coleman Junior Spectrophotometer. Enzymatic activity is expressed as the mg of acid soluble total N or "tyrosine" liberated per 100 cc of the protein solution obtained by subtracting the amounts found in the enzyme and substrate controls from that found in the

¹² Schmitz, A., *Z. physiol. Chem.*, 1936, **244**, 89.

¹³ Schmitz, A., *Z. physiol. Chem.*, 1937, **250**, 37.

¹⁴ Schmitz, A., *Z. physiol. Chem.*, 1938, **255**, 234.

¹⁵ Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

¹⁶ Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., MacDonald, A. H., and Minot, G. R., *J. Clin. Invest.*, 1945, **24**, 698.

¹⁷ Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

¹⁸ Edsall, J. T., *Advances in Protein Chem.*, 1947, **3**, 383.

¹⁹ Glazko, A. J., *J. Clin. Invest.*, 1947, **26**, 364.

²⁰ Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

TABLE I.
Control Studies on Protein Solutions and Casein Substrate.

2% protein solution	Principal components*	Acid soluble	
		Total N mg/100 cc†	"Tyrosine" mg/100 cc†
Fraction I	Fibrinogen	5.0	7.6
" II-III	Gamma and beta globulins	0.8	0.0
" IV-1	Lipids and alpha globulin	0.4	1.3
" IV-4	Alpha and beta globulins	0.0	0.0
" V	Albumin	0.0	0.0
Whole plasma		1.6	1.0
Casein		16.4	14.0

* Information directly from Dr. E. J. Cohn.

† After 48 hrs at 37.5°C, pH 7.1.

TABLE II.
Proteolytic Activity of Fraction I.

Protein solution			Exp.	pH	Incubation, hr	Net acid soluble	
						Total N mg/100 cc	"Tyrosine" mg/100 cc
2%	I	Run 25	A	6.7	48	150.4	
"	"	" "	B	6.3	"	113.6	120.9
"	"	" "	"	6.7	"	124.6	167.2
"	"	" "	"	7.1	"	9.4	24.4
"	"	" "	"	7.5	"	9.6	22.0
"	"	" "	"	7.9	"	7.6	21.8
0.25%	"	" "	C	6.7	"	4.4	2.4
1	"	" "	"	"	"	10.0	9.8
2	"	" "	"	"	"	23.0	27.6
"	"	" "	D	"	24		14.2
"	"	" "	"	"	33		21.2
"	"	" "	"	"	48		23.5
"	"	" "	"	7.5	24		17.0
"	"	" "	"	"	33		24.0
"	"	" "	"	"	48		27.4
"	I*	" "	"	6.7	33		-1.3
"	I*	" "	"	"	48		0.0
"	I	" †	E	"	"		76.1
"	"	" †	"	7.5	"		17.4
"	"	AVL-20	"	6.7	"		18.0
"	"	" "	"	7.5	"		108.8
"	"	" 186	"	6.7	"		92.0
"	"	" "	"	7.5	"		124.0
"	"	" 8/44	"	6.7	"		0.8
"	"	" "	"	7.5	"		1.0

* Inactivated for 3 min in 80°C water bath.

† Second sample of this preparation received from the Department of Physical Chemistry, Harvard Medical School.

enzyme-substrate mixture.

Results. Table I presents data which emphasize the necessity of both enzyme and substrate controls. It is shown that there were present in whole plasma and Fraction I, after 48 hours incubation alone, at pH 7.1, slight but significant amounts of acid soluble total nitrogen and "tyrosine." Similar studies were also made at pH 6.3, 6.7, 7.5, and 7.9 with almost identical results. It was also ob-

served that this amount in Fraction I was constant whether there was spontaneous clot formation and subsequent clot disappearance.²¹ There were traces of acid soluble N in Fractions II-III and IV-1 and none in Fractions IV-4 and V. These experiments could be duplicated. However, results with casein substrate alone were variable depend-

²¹ Shinowara, G. Y., unpublished observations, 1947.

ing on the particular lot of casein used. The data in Table I represent the highest values obtained. In this laboratory, it has been found that the acid soluble total nitrogen or "tyrosine" of Fraction I alone and of casein alone increases progressively with time of incubation.

Table II contains data on the proteolytic activity of Fraction I upon casein substrate. Experiment A was done 10 days; B, 25 days; C, 36 days; and D, 39 days after receiving the material labeled Run 25 from the Department of Physical Chemistry, Harvard Medical School. Experiment E was done one day after receiving a second shipment composed of material from different runs. With each experiment one or more of the other fractions and whole plasma were run as controls. The summary of results in mg net acid soluble "tyrosine" released from 2% casein by 100 cc of 2% solution of the specified protein at pH 6.3 to 7.9 and 48 hours incubation follows: whole plasma, 5.5 to 13.8; II-III, 6.4 to 12.0; IV-1, 0.0 to 7.8; IV-4, -4.5 to 0.4; and V, -10.2 to 0.0.

The data in experiments A, B, and E, Table II, definitely demonstrate the high degree of proteolytic activity of Fraction I, Run 25, although of a lesser degree, and demonstrate that the activity is apparently dependent upon the period of incubation and the concentration of the enzyme. The one sample of Fraction I, Run 8/44, which did not show enzyme activity differed from the other preparations of this fraction in several respects: It was pure white instead of light amber or gray in appearance; it was more than 90% insoluble in water or saline; and did not clot upon addition of thrombin. Runs 25, AVL, and 186 of Fraction I had clotting times with thrombin between 11 and 14 seconds, whereas Run 8/44, Fraction I, and samples of Fractions II-III, IV-1, IV-4, and V did not clot in 60 seconds.

In Experiment B, Table II, the greatest activity of Fraction I occurred at pH 6.7 and 6.3, but in Experiment D, there was approximately the same amount of proteolysis at pH 6.7 and 7.5. Variable results in pH studies with Fraction I, Runs 25 (second sample

received), AVL, and 186 were obtained in Experiment E. Therefore, from the data presented here, it can be stated that apparently the enzyme in Fraction I is active near neutrality. It is also evident from Experiment D that the enzyme activity was completely abolished by heating the 2% solution of Fraction I in an 80° bath for 3 minutes before mixing with the buffered casein substrate.

There was considerably less or no proteolytic activity in the whole plasma and the other fractions tested. No kinases or other activators were added, nor was there a previous treatment with chloroform or fibrin (calcium or thrombin) formation in these and other experiments.

Discussion. The direct demonstration of considerable proteolytic activity of Fraction I was made under analytical conditions intended to give minimal values: These included the use of both enzyme and substrate controls and of cold 20% trichloroacetic acid to precipitate the proteins at the end of the incubation period. By careful experiments, Hiller and Van Slyke²² showed that increasing the concentration of trichloroacetic acid increases the precipitation of non-protein nitrogen compounds.

The proteolytic activity of Fraction I can be explained on the basis that the low temperature-ethanol separation resulted in a preparation containing the zymogen and low, if any, concentration of inhibitor. This explanation would be based upon Christensen's¹⁰ hypothesis that the spontaneous activation, without streptokinase, of "plasminogen" occurs when the inhibitor content is low or absent or when the inhibitor has been inactivated by chloroform treatment of serum, resulting in a process which "appears to be autocatalytic in nature." His hypothesis is in agreement with his earlier observation⁸ that serum fractions prepared by dilution and acidification to pH 5.3 or by one-third saturation with ammonium sulfate may develop proteolytic activity without kinase or chloroform after a variable period of aging in the cold. The activity was

²² Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, **53**, 253.

measured by the gelatin-viscosity method. Christensen and MacLeod⁹ concluded that these solutions of inactive serum protease contained little or no inhibitor.

The higher activity of Fraction I at pH range 6.3-6.7 than at 7.1-7.9 in Experiment B, Table II requires some comment. In subsequent Experiments C and D, although significant degree of activity was still present, it had dropped appreciably. A possible explanation is that inadvertently the protein sample was left outside the refrigerator for several hours before Experiment C and this occurred when only a small amount remained in the sample bottle. Christensen¹⁰ reports that in the presence of active enzyme both the "plasminogen" and "plasmin" are destroyed. Nevertheless, the pH studies in experiments B and E may have significance in view of Grob's²³ observation on the shifting of pH optima of leucoprotease and trypsin in the presence of serum from pH 7-8 to pH 6-6.5 as a result of the progressively weakened inhibitor effect of serum upon exposure to pH below 6.5 or above 9.7.

Other preparations exhibiting activity without kinase include Schmitz's¹² calcium-fibrin adsorbed enzyme and the thrombin-fibrin adsorbed Fraction III-3.¹⁸ It appears from the data on these preparations and that reported here that the understanding of the significance of kinase and/or zymogen in the proteolytic system of human plasma *per se* requires

further experiments with inhibitor-free kinase, and purer preparations of zymogen, active enzyme, and naturally occurring inhibitors of plasma. Moreover, it is suggested that the liquefaction of a fibrin clot ("fibrinolysis"), the alteration of fibrinogen as measured by clotting time with thrombin ("fibrinogenolysis"), the change in gelatin viscosity, and the increase in acid soluble nitrogen are not representative of an identical enzyme system. In many of the incubation experiments with Fraction I alone "fibrinolysis" and "fibrinogenolysis" were observed while the acid soluble nitrogen did not increase appreciably; *i.e.*, not more than 2% of the total available nitrogen. A comparative study of the various criteria of proteolytic activity will be reported separately. True proteolytic activity (*i.e.*, the cleavage of the peptide bond) of all preparations of Fraction I, which are water and saline soluble, is demonstrated in this report.

Summary. The action of whole dried plasma and its primary fractions separated by low temperature-ethanol procedure on casein was investigated. No kinase was added and there was no previous treatment of the protein preparations with chloroform or any other agents. Proteolytic activity was demonstrated by determinations of acid soluble total nitrogen and "tyrosine."

Human plasma Fraction I contains a high degree of proteolytic activity. The enzyme is inactivated at 80°C and functions at near neutrality.

²³ Grob, D., *J. Gen. Physiol.*, 1946, **29**, 219.

Effect of Histolyticus Infection and Toxin on Transplantable Mouse Tumors.*

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The treatment of experimental and human tumors with bacterial infections and bacterial products began with the work of Busch, almost 80 years ago.¹ Since then, the use of bacterial products in the treatment of clinical and experimental cancer has received the attention of many investigators, including Fehleisen,² Bruns,³ Coley,⁴ Gratia and Linz,⁵ Duran-Reynals,⁶ Andervont,⁷ and Shear and his co-workers.⁸ Brues and Shear⁹ reported the treatment of 4 patients with intramuscular injections of a polysaccharide from culture filtrates of *Serratia marcescens* (*B. prodigiosus*), which had previously been shown to produce hemorrhage in and partial or complete destruction of sarcomas in mice.

In previous experiments carried out by two of the present authors, it was demonstrated that in experimental *Cl. welchii* infections in mice,¹⁰ and to an even more marked degree in experimental infections with *Cl. histolyticum*,¹¹ the systematic administration

of the specific antitoxin counteracts the toxemia without preventing the early development of the large local lesions produced by the lytic action of toxins of the gas gangrene group of anaerobes. Accordingly, it seemed reasonable to undertake an investigation designed (a) to destroy tumor tissue by infection with histolyticus spores, to control the resulting toxemia by means of a systemic administration of antitoxin and, finally, to control the infection itself by means of penicillin; (b), to destroy tumor tissue by repeated local injections of small doses of histolyticus toxin; and (c) to test the effect of histolyticus toxin on sarcoma, carcinoma and normal tissues cultivated *in vitro*. Although the results of the experiments that were carried out do not warrant the use of histolyticus infection, or of partially purified histolyticus toxin, in the treatment of human tumors, it is believed that they are of sufficient theoretical interest to justify the present report.

1. *Sarcoma treated with viable histolyticus spores, followed by antitoxin and penicillin. Exp. A.* The first experiment included thirty-three C57 black mice, 18 of which were inoculated with a rapidly growing, transplantable fibrosarcoma (Bar Harbor: L946AII). Of the sarcoma mice, 15 were infected with a spore suspension of *Cl. histolyticum* contained in 0.1 ml of a 5% calcium chloride solution that was injected directly into the developing tumors 8 days after tumor inoculation. Ten of these mice were subsequently treated either with antitoxin (Group 1, 5 animals) or with sodium-penicillin (Group 2, 5 animals) in order to control the infection. Five tumor mice infected with the spore suspension were otherwise untreated (Group 3). Three tumor mice received no treatment whatsoever (Group 4).

* This investigation was supported, in part, by a grant from the Ontario Cancer Treatment and Research Foundation.

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2 Fehleisen, *Deutsch. med. Woch.*, 1882, **8**, 553.

3 Bruns, P., *Beitr. klin. Chir.*, 1888, **3**, 443.

4 Coley, W. B., *Ann. Surg.*, 1891, **14**, 199; *Glasgow Med. J.*, 1936, **126**, 49, 128.

5 Gratia, A., and Linz, R., *C. R. Soc. biol.*, 1931, **108**, 427.

6 Duran-Reynals, F., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 341; 1935, **32**, 1517.

7 Andervont, H. B., *Am. J. Cancer*, 1936, **27**, 77.

8 Shear, M. J., *et al.*, *J. Nat. Cancer Inst.*, 1943, **4**, 81, 99, 107, 123; 1944, **4**, 461.

9 Brues, A. M., and Shear, M. J., *J. Nat. Cancer Inst.*, 1944, **5**, 195.

10 Siebenmann, C. O., and Plummer, H., *J. Pharm. and Exp. Therap.*, 1945, **83**, 71.

11 Siebenmann, C. O., and Plummer, H., unpublished experiments.

TABLE II.
Effect of Histolyticus Infection on Sarcoma in Mice (Exp. B).

Group Infection Treat- ment*	Sarcoma bearing mice						Normal mice					
	1		2		3		4		5		6	
	<i>Cl. histolyticum</i> Spores Histolyticus antitoxin (s.c.)	Day of death†	Size of sarcoma	Day of death	Size of sarcoma	Day of death	<i>Cl. histolyticum</i> Spores Penicillin (i.m.)	Day of death	Size of sarcoma	Day of death	<i>Cl. histolyticum</i> Spores Penicillin (i.m.)	Day of death
Mouse No.												
1	3 small (L)†	2 medium (L)	2 medium (L)	2 medium (L)	1 small	66§	normal	2 lesion +	1 lesion +			
2	4 medium (L)	6 large	6 large	2 small	1 "	66§	"	2 "	2 "			
3	4 large	6 "	6 "	2 "	6 large	66§	"	47 "	3 "			
4	5 "	7 "	7 "	2 medium (L)	7 "	66§	"	66§ lesion + (healed)	3 "			
5	6 medium (L)	7 small	7 small	3 small	10 "	66§	"	66§ normal	3 "			
6	8 "	8 "	8 "	3 "	10 "	66§	"	66§ "	4 "			
7	15 large	10 large	10 large	3 "	11 "	66§	lesion + + (healed)	66§ lesion + (healed)	5 "			
8	22 medium (L)	11 large	11 large	5 "	11 "	66§	normal	66§ "	5 "			
9	25 "	12 "	12 "	8 large	12 "	66§	"	66§ "	5 "			
10	32 small (L)	12 "	12 "	12 "	12 "	66§	lesion + + (healed)	66§ "	7 "			

* For dosage, see text.

† Number of days after spore infection.

‡ (L) denotes lysis of tumor.

§ Mouse apparently healthy when sacrificed.

|| Small lesion +; medium lesion + +; large lesion + + +.

a small tumor that grew to full size and resulted in death on the 37th day.

The 5 non-tumor mice that were infected with spore material and treated subsequently with antitoxin (Group 5) showed healed lesions at the time the experiment was terminated on the 37th day. Of the 5 non-tumor mice that were infected with spore material and treated subsequently with penicillin (Group 6), one showed a healed lesion at the time of death, from unknown causes, on the 8th day, whereas the others, also with healed lesions, survived until the termination of the experiment. None of the 5 non-tumor mice infected with spore material without further treatment (Group 7) survived more than 3 days. All showed extensive lysis of the muscles of the infected leg.

Exp. B. As a further means of studying the effect of histolyticus infection on mouse sarcoma, a second experiment was carried out. Except that the groups were larger, this experiment was similar to the previous one in all essential details.

Results (Table II). The sarcoma grew faster in this experiment and killed all the control mice (Group 4) by the 12th day. Of the 10 tumor mice that were infected with histolyticus spores and treated subsequently with antitoxin (Group 1), 4 survived the untreated tumor bearers. In order to suppress any residual histolyticus infection in these 4 survivors, penicillin treatments were begun on the 8th day after infection. The dosage (intramuscular injections of 250 units) ranged from 3,250 units (mouse No. 7) to 6,500 units (mouse No. 10) and depended upon the length of time each mouse survived. All 4 mice finally developed tumors, though in 3 the sarcoma showed definite signs of lysis at the time of death. The mouse that lived longest (mouse No. 10) outlived the last surviving sarcoma-control by 20 days.

II. Carcinoma treated with viable spores, followed by antitoxin and penicillin. This experiment, which was carried out simultaneously with Exp. A, and in essentially the same manner, included 33 first generation hybrid mice obtained by mating C57 black females with C3H males. The tumors consisted of

transplantable adenocarcinomas (Bar Harbor: E-0771).

Results. Unlike the sarcomas treated in Exps. A and B, the carcinomas showed little evidence of tumor regression following the injection of spore material. With the exception of 2 mice in the group of tumor bearers that received spore material without subsequent treatment, all tumor-bearing mice survived for at least 13 days and developed tumors of considerable size.

III. Sarcoma treated with toxin. This experiment included thirty-five C57 black mice, 20 of which were inoculated with the fibrosarcoma (Bar Harbor: L946AII) already mentioned. Of the sarcoma mice, 15 were treated with the toxin of *Cl. histolyticum* administered on the 6th, 8th, 10th and 13th days following tumor inoculation. In 5 of these mice (Group 1), the toxin was introduced directly into the tumor in 4 injections of 1.5 mg each. In 5 mice (Group 2), the same amounts of toxin were injected subcutaneously around the base of the tumor. For the remaining 5 (Group 3), which also received subcutaneous injections around the base of the tumor, the initial injection of toxin consisted of twice the usual amount. Five sarcoma mice (Group 4) received no treatment whatsoever. The 15 control mice that did not receive tumor inoculations were divided into 3 groups of 5 mice each. These groups received the same amount of toxin, respectively, as Groups 1, 2 and 3 of the tumor-bearing mice.

The toxin consisted of a powdered, dry preparation provided by the National Institute of Health (Bethesda, Md.). It was dissolved in saline, distributed in small tubes and kept frozen in a CO₂-ice box until used.

Results. The 5 sarcoma bearers that received no treatment died between the 10th and the 13th day after tumor inoculation; and those that died after the 10th day showed large tumors. There were no deaths among the 15 non-tumor control mice that received toxin injections, although 10 of them developed lesions that eventually healed. Of the 5 tumor-bearing mice that were injected with toxin introduced directly into the devel-

oping tumors (Group 1), 2 died on the 7th day following tumor inoculation (1 day after the beginning of treatment) and the others survived for 13, 14 and 29 days, respectively. The 5 tumor bearers that were injected with toxin introduced about the base of the tumor (Group 2) died between the 14th and 31st days following tumor inoculation. Of the 5 tumor bearers that were injected with toxin introduced subcutaneously about the base of the tumor, but with a larger initial dose (Group 3), one died on the 7th day after tumor inoculation, and 4 survived from 32 to 36 days. Two mice of Group 2 and two mice of Group 3 showed tumors that were only about half the size of those observed in the untreated control mice, whereas the other mice of these groups developed large tumors. In Group 1, in which the toxin was injected directly into the tumors, the 3 mice that survived the treatment for 7 days or more showed an even greater reduction in tumor size.

IV. Carcinoma treated with toxin. Although a large series of experiments were made in an effort to test the effect of histolyticus toxin on carcinoma, only one will be reported in detail. This experiment included 16 first generation hybrid mice obtained by mating C57 black females with C3H males. Twelve of these mice were inoculated with a slow-growing, transplantable adenocarcinoma that had arisen spontaneously in a female of the C3H stock. Of the 12 tumor-bearing mice, 4 were treated with a total of 2.5 mg of histolyticus toxin that was introduced directly into the tumor in 8 injections over a period of 20 days, 4 were treated with the same amount of toxin that was introduced intramuscularly but into the leg opposite the one bearing the tumor, and 4 were left untreated. Four control mice without tumors were treated with a total of 4.0 mg of toxin given in 8 injections over 20 days. This was an amount of toxin in excess of that used in treating the tumor-bearers.

Results. On the 7th day following the beginning of toxin treatment (30 days after tumor inoculation), there were 3 deaths in the group that had been injected with toxin

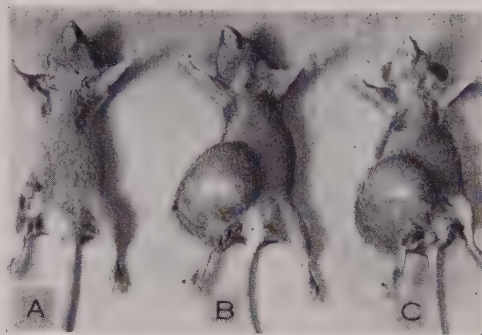


FIG. 1.

Hybrid mice (C57 black x C3H) inoculated with slow-growing, transplantable adenocarcinoma and subsequently treated with 2.5 mg of histolyticus toxin in 8 injections over period of 20 days. (A) Toxin injected directly into tumor, which weighed 0.25 g when photographed; (B) Control, untreated; (C) Toxin injected intramuscularly into leg opposite the one bearing the tumor.

introduced directly into the tumors. At the time of death the tumors were bean-sized, 2 with slight lesions and one with a healed lesion. The remaining mouse of the group carried a small tumor that weighed 0.25 g at the termination of the experiment (Fig. 1A) 44 days after the tumor had been initiated and 21 days following the beginning of toxin treatment. The 4 tumor-bearing mice that had not been treated with toxin developed large tumors that averaged 7.25 g in weight at the termination of the experiment (Fig. 1B). The 4 tumor bearers that had been treated with toxin injected intramuscularly into the opposite leg also developed large tumors that averaged 6.56 g at the termination of the experiment (Fig. 1C).

V. *In vitro* studies. An effort was made to test the effect of histolyticus toxin on sarcoma, carcinoma and normal tissues cultivated *in vitro*, and also, by the same means, to demonstrate the protective action of anti-toxin. Kidney was chosen as the normal tissue because it yields, in tissue culture, an abundant growth of both epithelium and connective tissue. The majority of the experiments were carried out in large Carrel flasks (5 cm in diameter) in which 3 or more fragments of sarcoma (Bar Harbor, L946-AII), of carcinoma (Bar Harbor, E-0771), and of young, adult mouse kidney were em-

bedded in 2.0 cc of medium consisting of 0.5 cc of chicken plasma, 0.2 cc of toxin, 0.4 cc of antitoxin and 0.9 cc of a mixture comprised of 40% horse serum (heated $\frac{1}{2}$ hr. at 56°C), 40% Earle's solution, 20% chick embryo tissue juice and sufficient phenol red to make a final concentration of 0.005%. In the control cultures, the toxin and antitoxin were replaced by Earle's solution. The cultures were adjusted with gas mixtures to pH 7.2 and incubated without further treatment for 6 days.

When the medium contained both toxin and antitoxin, the toxin used in the various cultures of a typical series ranged in amount from 0.1 mg to 2.0 mg per cc. When the toxin was used alone, the amounts ranged from 0.01 mg to 1.0 mg per cc. Antitoxin, when present, was in the amount of 0.2 units per cc.

Results. In general, it may be said that the carcinoma was more resistant to the action of the toxin than sarcoma, just as normal epithelium seemed to be more resistant than stroma cells or fibroblasts. And normal and cancerous epithelium were equally resistant to concentrations of toxin that produced severe damage to the sarcoma cells.

In effective concentrations of toxin, the fibroblast-like sarcoma cells in the zone of outgrowth (or outward migration) rounded up, their nuclei and cytoplasm underwent a pronounced shrinkage, and they proceeded to disintegrate. But the cells of the explant were also affected. They too rounded up, underwent shrinkage and became dissociated into separate entities quite devoid of visible intercellular connections.

Discussion. With reference to the action of histolyticus infection on transplantable tumors, two possible effects were foreseen. The local infection of the tumor tissue might take the course of an acute anaerobic infection, which leads to toxemia and results in the early death of the animal host. Or, the local infection might, apart from any injurious effect on the animal, cause lysis of the tumor tissue and thereby prolong the life of the animal. And because earlier observations on histolyticus infections had shown that antitoxin

counteracts the systemic effect of the toxin without eliminating, necessarily, its lytic action at the site of infection,¹¹ it seemed reasonable to study the effect of the antitoxin-treated infection on developing mouse tumors.

Both experiments in which sarcoma was treated with spores showed that the uncontrolled infection led more rapidly to the death of the animal than when the tumors were left untreated. The same held true for spore infected tumors treated with penicillin. The effect of the antitoxin treatment, however, was to prevent or postpone death from toxemia; and, as had been expected, it left enough of the local infection to cause a more or less marked lysis of the tumor tissue, thereby prolonging the life of some of the tumor bearers considerably beyond the life span of the non-infected tumor controls.

In order to determine whether, in such survivors, the histolyticus infection had completely destroyed all tumor tissue, the infection was finally eliminated by means of prolonged penicillin therapy. But none of the antitoxin treated mice, subsequently given penicillin for this purpose, survived the test; sooner or later they all developed sarcomas from which they died.

It has been noted that in the experiment in which carcinoma was treated with spore material followed by antitoxin and, finally, penicillin, these tumors, unlike the sarcomas, showed little or no regression. But it was impossible to determine, from the limited observations that were made, whether the greater resistance of the carcinoma was due to a greater resistance of the constituent cells or to the peculiar structure of the tumor itself. From the tissue culture experiments that were made with toxin, it would seem that the carcinoma is actually more resistant than the sarcoma, and that the greater susceptibility of the sarcoma may possibly be due to a greater looseness in the architecture of this type of tumor. In the cultures, the sheets of epithelial cells that grew out from the fragments of carcinoma survived concentrations of toxin that were definitely injurious to the sarcoma cells. But normal epithelium, growing side by side with the malignant epithelium,

was likewise resistant to concentrations of toxin that killed the sarcoma cells. The spindle-like sarcoma cells grow in an open meshwork, whereas carcinoma and normal epithelium grow in a compact mosaic in which the individual cells are far less vulnerable to outside influences.

In so far as the animal experiments with toxin are concerned, there is little that need be added to the observations already made. Just as the antitoxin-controlled histolyticus infection has, under certain conditions, a retarding influence on the development of the tumors, so also are the tumors susceptible to carefully regulated injections of toxin, the amount of which must be sufficient to produce lysis of the tumor but not enough to cause the death of the host. But regardless of how completely a tumor may seem to have regressed, it is almost certain to recur once the treatments have ceased. To be sure, the absorption of toxin does seem to be more rapid in tumor tissue than in normal tissue. Aside from the direct effect of the toxin on the tumor cells themselves, there is also damage to the young, actively proliferating vascular system of the tumor, and this results in hemorrhage and necrosis and the general destruction of the tumor mass. And as further evidence that the effect of histolyticus toxin on tumor cells is not a specific one, systemic injections of toxin at concentrations below the level that is injurious to the host have no effect on the tumors, just as toxin injected around the base of a developing tumor produces less lysis of the tumor than toxin injected directly into the tumor mass.

The main difficulty in attempting to evaluate the effect of histolyticus toxin on experimental tumors, in the present experiments, is the fact that the minimal effective dose approximated, very closely, the lethal dose.

For further study, a more highly purified preparation of toxin would be required, and an attempt should be made to isolate, from the crude toxin, a factor that would be both highly histolytic and of low systemic toxicity.

Summary. When transplantable mouse sarcomas were infected experimentally with *Cl. histolyticum*, and the infection controlled by systemic injections of histolyticus antitoxin, the life span of some of the animals was prolonged for as long as 20 days beyond that of the non-infected tumor bearers. But in no instance did the infection completely destroy the tumor tissue. All surviving mice, treated eventually with penicillin in order to eradicate residual infection, developed large sarcomas. Under similar conditions, no such temporary regression was observed for transplantable mouse carcinomas.

When a study was made of the effect of histolyticus toxin on transplantable mouse tumors, it was found that systemic (intramuscular) injections of toxin were without effect, whereas repeated local injections of toxin, given either directly into the tumor mass or subcutaneously around the base of the tumor, resulted in marked regression of tumor tissue. But for the toxin preparations used, the effective dose approximated, very closely, the lethal dose, particularly when the toxin was injected directly into the tumor mass. In no instance was there a permanent regression of the tumor.

In tissue culture, carcinoma cells were more resistant to the action of histolyticus toxin than sarcoma cells, just as normal epithelium seemed to be more resistant than stroma cells or fibroblasts. But normal and cancerous epithelium were equally resistant to concentrations of toxin that produced severe damage to sarcoma cells.

Comparison of Two Types of Permanent External Bile-fistula Dogs for Studying Liver Function.

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Studies of hepatic physiology involving the use of dogs with the usual types of permanent external bile fistula are open to criticism because of the great difficulty of maintaining normal liver function in such animals except for short intervals. This is emphasized by the recent report by Drill¹ and is borne out by our experience during the course of previous studies of the excretion of bromsulfalein in bile.^{2,3,4} Only 6 of 22 bile-fistula dogs prepared for this purpose proved to be satisfactory for the establishment of criteria for normal excretion of the dye. The purpose of the present communication is to indicate the usefulness of another type of fistula dog for such studies.

Materials and Methods. The two types of permanent biliary fistula employed were the standard type (Rous-McMaster⁵) as modified by Beerman *et al.*⁶ and the type in which a Thomas intestinal fistula is placed opposite the papilla of Vater.^{7,8} In the former, following cholecystectomy, a rubber tube was placed in the cystic duct with the common duct tied, or in the common duct with the cystic duct tied, and brought out through a stab wound in the abdominal wall. Bile drained into an

attached rubber bag and was reintroduced into the duodenum through a small indwelling duodenal catheter. These dogs recovered slowly after operation, required a special diet and daily care. Because they were prone to develop biliary obstruction they usually survived for only a few weeks or months. As a rule the obstructive phenomena were not pronounced and were unrecognized until the excretion of bromsulfalein in the bile was tested or jaundice developed.

The Thomas intestinal fistula was prepared following cholecystectomy by placing a permanent metal cannula in the duodenum opposite the papilla of Vater. With the cannula open a special glass catheter^{9,10} (modified by Thomas from those originally devised by Scott), was introduced when desired into the ampulla of Vater for the drainage of bile. Upon termination of the experimental period the catheter was withdrawn and the cannula closed, which prevented further loss of intestinal contents. These dogs recovered promptly from the operation, did not require special diet or care and survived for long periods, some for over 2 years, without developing biliary obstruction.

Studies were made on 7 dogs with standard type fistulas (11 tests) and on 3 dogs with Thomas type fistulas (19 tests). All of these dogs were given intravenous injections of 2 mg bromsulfalein per kilo of body weight. Four additional Thomas-type fistula dogs were studied by comparing the excretion of bromsulfalein injected in quantities of 2 mg and 5 mg per kilo of body weight.

Bile was collected in 15-minute periods for 2 hours subsequently. Determinations were

¹ Drill, V. A., Annegers, J. A., Snapp, E. F., and Ivy, A. C., *J. Clin. Invest.*, 1945, **24**, 97.

² Cantarow, A., and Wirts, C. W., Jr., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **47**, 252.

³ Wirts, C. W., Jr., and Cantarow, A., *Am. J. Dig. Dis.*, 1942, **9**, 101.

⁴ Cantarow, A., and Wirts, C. W., Jr., *Am. J. Dig. Dis.*, 1943, **10**, 261.

⁵ Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, **37**, 11.

⁶ Berman, A. L., Snapp, E., Ivy, A. C., Atkinson, A. J., and Hough, V. S., *Am. J. Dig. Dis.*, 1940, **7**, 333.

⁷ Thomas, J. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 260.

⁸ Snape, W. J., and Thomas, J. E., *Fed. Proc.*, 1945, **4**, 66.

⁹ Hart, W. M., and Thomas, J. E., *Gastroenterol.*, 1945, **4**, 409.

¹⁰ Scott, V. B., Collingnon, U. J., Bugel, H. J., and Johnson, G. C., *Am. J. Physiol.*, 1941, **134**, 208.

TABLE I.
Excretion of Bromsulfalein in the Two Types of Fistula-dogs Using 2 mg of Dye per Kilo of Body Weight.

	Standard fistula dogs		Thomas-type fistula dogs
	Unselected	Selected	
Range of conc. (mg dye per 100 cc bile)	1 to 476	1 to 532	6 to 540
Avg % excreted			
1 hr	5.9	43.6	45.7
2 hr	18.1	65.0	75.0
Vol. of bile (cc) per hour	7.4	—	7.7

Excretion of Bromsulfalein in Thomas-type Fistula Dogs Using 2 mg and 5 mg of Dye per Kilo of Body Weight.

	5 mg dosage	2 mg dosage
Range of conc. (mg dye per 100 cc bile)	5 to 1430	6 to 540
% excreted		
1 hr	51.2	45.7
2 hr	64.1	75.0

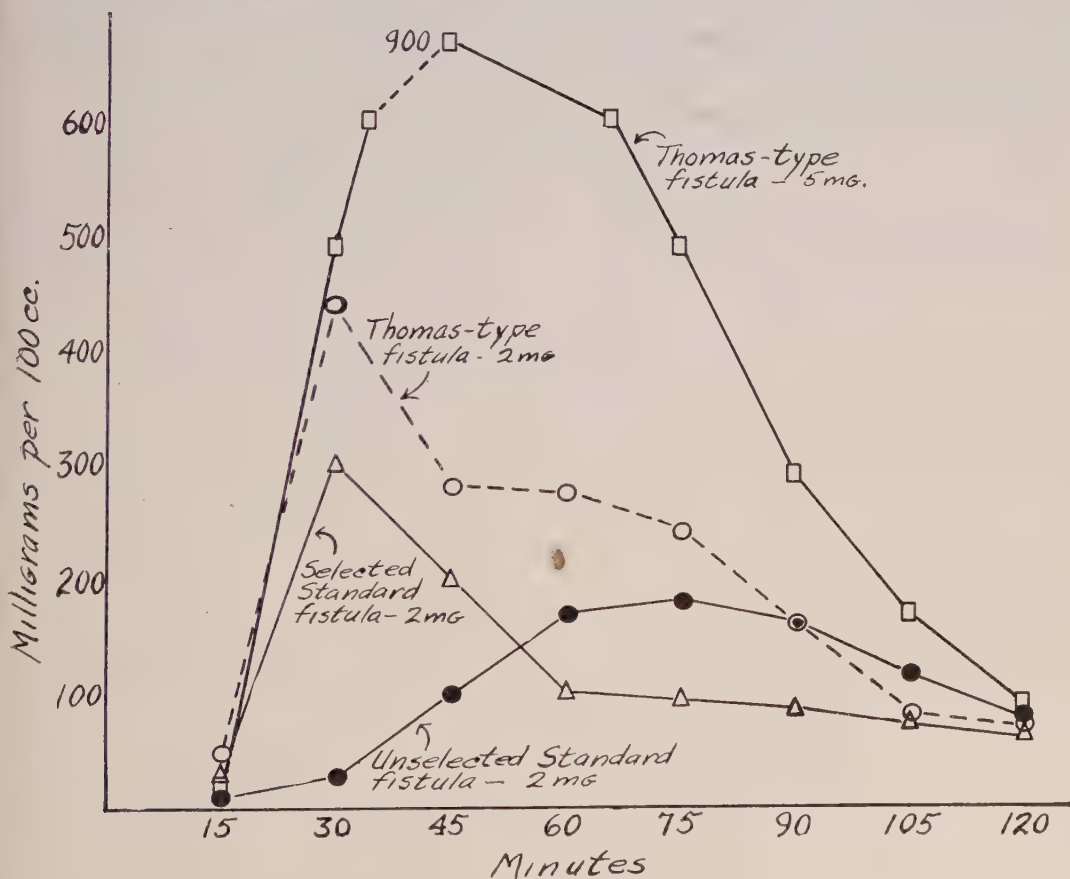


FIG. 1.

Curves of concentration of bromsulfalein in the bile of bile fistula dogs, after injection of 2 mg and 5 mg per kilo of body weight.

made of the concentration and amount of bromsulfalein in each sample. The following criteria were used as a basis of comparison of the two groups of animals: (1) completeness of removal of dye from the blood; (2) rate of entrance of dye into the bile; (3) time of attainment of maximum concentration of dye in the bile; (4) curve of excretion of dye in the bile; (5) range of concentration of dye in the bile and (6) total dye excretion in the bile within one- and 2-hour periods following injection.

Prior to initiating a series of liver function studies the two types of permanent biliary fistula dogs were compared for suitability by observing their prompt convalescence from operation, maintenance of good general physical condition and liver function. The liver function was tested by estimating the quantity and rate of excretion in the bile of intravenously injected bromsulfalein and, in some animals, estimating the quantity of dye retained in the blood according to a method previously described.²

Results. The pertinent data are summar-

ized in the table and figure. No abnormal retention of dye in the blood was observed in the Thomas-type fistula dogs; abnormal retention occurred in 5 of 6 standard fistula dogs. Similarly, the unselected dogs with the Thomas-type fistula were superior to unselected dogs with the standard type fistula when judged by the other criteria enumerated above. Dogs in the latter group selected on the basis of satisfactory bromsulfalein excretion approached the former in this regard.

Summary and Conclusions. Evidence has been presented to indicate that dogs with the Thomas-type bile fistula are much more satisfactory than those with the Rous-McMaster-type fistula for studies of liver function, particularly those involving collection and examination of bile. Data are presented regarding the normal biliary excretion of bromsulfalein following intravenous injection of 2 mg and 5 mg of the dye per kilogram of body weight.

The authors are indebted to Dr. J. Earl Thomas for advice and assistance in the preparation of the bile-fistula dogs used in these studies.

16126 P

Urticarial Hypersensitivity to Sunlight.

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Urticarial hypersensitivity to sunlight or solar urticaria is a well recognized disease entity.¹ The reaction is analogous to the urticarial hypersensitivity reactions to foods, drugs, and such physical agents as mechanical, cold or heat stimuli. These reactions have all the characteristics of Thomas Lewis' "triple response".² It is generally assumed that in this type of hypersensitivity the antigen-antibody reaction leads to wheal formation

via the liberation of histamine. Solar urticaria has been regarded as one form of the "physical allergies" in which an antigen or allergen is produced by action of a physical agent. Recently two types of solar urticaria have been described.^{3,4} In one type the sensitivity spectrum is between λ 4000-5000 Å and in the other type it is below λ 3700 Å.

Experimental. In this clinic 2 patients were studied who responded to sunlight exposure with severe urticarial reaction. In both cases

¹ Blum, H. F., *Photodynamic Action and Diseases Caused by Light*, New York, Reinhold Publ. Corp., 1941.

² Lewis, T., *Blood Vessels of the Human Skin and Their Response*, London, Shaw and Sons, 1927.

³ Abramson, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 410.

⁴ Blum, H. F., Baer, R. L., and Sulzberger, M. B., *J. Invest. Derm.*, 1946, **7**, 99.

it was possible to establish the spectral sensitivity: (1) by radiating the skin of the patients through a set of Corning glass filters and (2) by use of a monochromator which made it possible to project single lines of the mercury arc spectrum on the skin. In both cases the range of sensitivity was found to be between 2967 and 3341 Å with a maximum sensitivity at 3131 Å. Thus these 2 patients belong to the type $< \lambda$ 3700 Å.⁴

The urticarial sensitivity could be passively transferred. By injecting the patients' serum into the skin of normal individuals and irradiating the injection site with the active wavelengths, a "triple response" could be elicited. The transfer site began to manifest sensitivity within 30 minutes after injection and remained sensitive for several days although the sensitivity decreased with time. Normal control sera never caused such reactions.

The incidence of successful passive transfers was much higher with the serum of the more sensitive patient than with the serum of the less sensitive one. When large surfaces of the body were exposed to ultraviolet light prior to withdrawal of blood for passive transfer experiments, the incidence of positive transfers was greatly increased.

Studies of the properties of the transferable sensitizing agent in the serum revealed that it decreased in potency with storage at ice-box temperature. After 8 days cold storage of otherwise potent serum, samples gave only weak or questionable passive transfer tests. The sensitizing agent proved to be heat labile and was inactivated at 56°C in 1/2 hour. Also, the agent was inactivated by irradiation with ultraviolet light. Evidence that the photosensitizing agent is a large molecule, probably of colloid nature, is suggestive since it failed to dialyze through a semi-permeable membrane.

Early morning gastric analysis following radiation of large areas of the patients' skin showed a rise in total acidity in both patients.

A number of substances with absorption spectra similar to the sensitivity spectrum of

the patients were incorporated into ointments and tested for protective action against the active rays. Some protection was obtained by using an ointment containing 30% G-Salt (sodium salt of 2-naphthol-6,8-disulfonic acid).

Greater protection was achieved by the oral administration of antihistaminic drugs, benadryl and pyribenzamine. By virtue of the protective action of these "antihistamines" it was possible to subject the patients to gradually increasing generalized ultraviolet light exposures.⁵ By this treatment the skin acquired such tolerance that the administration of "antihistamines" could be discontinued. This gradual increase in tolerance to the active wavelengths was purely a local effect since no protection was achieved on skin areas which were kept covered during the course of ultraviolet therapy. The protection may have resulted from pigmentation and/or the thickening of the horny layer of the skin. Both of these physiologic reactions to sunshine hinder the penetration of the active wavelengths.

Discussion. The narrow and highly specific range of the active wavelengths in this condition together with the passive transferability of the sensitivity to normal individuals with the patients' serum strongly supports the theory of Sulzberger and Baer.⁶ According to these authors, the absorption of the specific wavelengths causes the formation of a photochemical product in everybody's skin; however, patients with solar urticaria develop a sensitivity by forming antibodies against this normal metabolite.

Summary. In 2 cases of solar urticaria the maximum sensitivity was found to be at 3131 Å. Studies of the photosensitizing agent in the patients' serum support the conception that this condition is due to sensitization to a physiological radiation product in the skin.

⁵ Rubin, L., Beal, P. L., and Rothman, S., *J. Invest. Derm.*, 1947, **8**, 189.

⁶ Sulzberger, M. B., and Baer, R. L., *J. Invest. Derm.*, 1945, **6**, 345.

Studies on Uterine Metabolism. I. Adenosinetriphosphatase Activity of Smooth Muscle.

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The activity of the adenosinetriphosphatase (ATP-ase) enzyme system has been measured in many tissues other than skeletal muscle. DuBois and Potter¹ have indicated that an unidentified smooth muscle yielded, in their tests, about one-third of the ATP-ase activity of striated muscle. We have investigated rather extensively the ATP-ase system in the smooth muscles of the rat, in particular the uterus: ATP was prepared as the dicalcium salt according to Kerr.²

The reaction systems were incubated at 37°C for 15 minutes in a final volume of 3 ml, and contained one ml of a tissue homogenate¹ in 0.1 M veronal-HCl buffer of pH 8.4; one ml of a 10^{-3} molar solution of ATP and one ml of veronal-HCl buffer. Magnesium ions were present in the system in a final concentration of 10^{-4} molar.³ Blanks were run with ATP alone, and also with tissue in the absence of ATP. Addition of other substances was compensated for in volume by a corresponding reduction in the volume of buffer. The liberated phosphate was determined by the method of Fiske and SubbaRow.⁴

Table I expresses the results found for ATP-ase activity of smooth and skeletal muscles of adult Sprague-Dawley rats. These were all used in the metestrus stage as determined by the daily vaginal smear technique. Varying amounts of tissue were used to insure a linear relationship between tissue weight and enzyme concentration.

The larger variations in the smooth muscle

determinations were thought to be due to the water content fluctuations which these tissues undergo, depending upon their immediate physiological states. However, calculation of the data on the basis of dry weights yielded similarly extensive variations. It must, therefore, be assumed that these changes arise through some other mechanism.

It is significant, however, that the smooth muscles in these systems exhibit a uniformly higher activity than striated muscle. In later experiments striated muscle was often tested at the same time as smooth muscle from the same animal; the above differences in enzymatic activity were always observed to hold true.

The contractile system of smooth muscle remains considerably more obscure than that of skeletal muscle. If the ATP-ase system of smooth muscle is to follow the same myosin-adenosinetriphosphate changes postulated for striated muscle^{5,6} the sensitivities of these systems to chemical mediators such as acetylcholine and epinephrine should be similar. Ziff⁷ has reported that acetylcholine, epinephrine and eserine are without effect on the hydrolysis of ATP by myosin, which we assume he obtained from skeletal muscle. We are able to corroborate his findings for striated muscle and extend them to uterine muscle. The concentration of the various mediators used is of the same order of magnitude as that employed to elicit physiological responses *in vitro*. The data are given in Table II.

These variations are not considered significant in the light of changes in enzymatic

¹ DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

² Kerr, S. E., *J. Biol. Chem.*, 1941, **139**, 121.

³ Singher, H. O., and Meister, A., *J. Biol. Chem.*, 1945, **159**, 491.

⁴ Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

⁵ Engelhardt, V. A., *Advances in Enzymology*, 1946, **6**, 147.

⁶ Szent-Györgyi, A. von, *Bull. soc. chim. biol.*, 1943, **25**, 242.

⁷ Ziff, M., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 249.

TABLE I.
ATP-ase Activity of Smooth and Skeletal Muscle.
Values expressed as μg P liberated in 15 min.

Tissue homogenate	No. of trials	Per mg fresh tissue		Per mg dry tissue	
		Avg	Range	Avg	Range
Skeletal muscle (Sartorius)	9	8.1	(5.8-13.2)	33.7	(25.0- 56.0)
Intestine (1st loop of duodenum)	6	18.0	(14.0-24.2)	61.8	(46.9- 78.0)
Bladder	4	20.9	(16.6-23.2)	81.8	(73.0-103.0)
Uterus	6	20.8	(16.8-24.2)	89.0	(62.6-110.0)

TABLE II.
Influence of Chemical Mediators on ATP-ase Activity of the Uterus.

Substance	Final conc.	Estrus stage	Avg % change in ATP-ase activity of uterus
Acetylcholine	7×10^{-9} M	Metestrus	+1.5
		Diestrus	-4.4
Epinephrine	1×10^{-7} M	Metestrus	+6.3
		Diestrus	+2.4
Eserine	4×10^{-9} M	Diestrus	+1.4

activity of tissues to which the above substances have not been added. As far as these *in vitro* tests go it may be concluded, therefore, that in skeletal and smooth muscle, these mediators do not effect the ATP-ase system *per se*. The activation of ATP-ase by acetylcholine, described by DuBois and Potter⁸ was carried out in a system in which the calcium concentration was a factor which is not the case in these experiments.

Consistent changes in the activity of ATP-ase in smooth or skeletal muscle could not be demonstrated with histamine, pitocin or ammonium chloride. Ethyl alcohol (0.7 M) inhibited the system slightly but this is probably due to its protein denaturant properties. Engelhardt,⁵ Ziff,⁷ and others⁹ have indicated that the inhibitory effect of fluoride is only as great as its removal of calcium ions from the medium. In the magnesium activated system we have observed inconsistent effects of fluoride on striated muscle. Uterine muscle ATP-ase, however, is usually inhibited, as shown in Table III.

The ATP-ase activity of the uterus has led us to investigate the effect of the reproductive cycle on such activity. Because of the water shifts it seemed best to calculate

TABLE III.
Effect of 3.3×10^{-3} M Fluoride on ATP-ase System of Smooth Muscle.

Stage of cycle	% change of ATP-ase activity
Estrus	-25.8; -37.8
Metestrus	-15.5
Diestrus	-17.4; -40.7
Proestrus	-18.6

activity on the basis of dry weight of the tissue used. Later experiments have also included per cent nitrogen as a factor. The results from preliminary experiments do not lend themselves to an interpretation of variation with the cyclical changes, as indicated in Table IV.

Representative steroid hormones were added to the incubate in concentrations of between 10^{-4} to 10^{-6} molar. Additions were made in a small amount of alcohol, and alcohol controls run simultaneously. Estrone, estradiol and progesterone were without appreciable effect on the uterus in the metestrus stage; the latter two also had negligible effect during diestrus. In a small number of cases testosterone seemed to inhibit the ATP-ase activity of skeletal muscle; its effect on uterine muscle appeared to be much smaller.

Summary. The ATP-ase activity of smooth muscle is found to be considerably higher than that of striated muscle. It is unaffected by dilute concentrations of chemical mediators. Fluoride apparently has an inhibitory

⁸ DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **148**, 451.

⁹ Polis, B. D., and Meyerhof, O., *J. Biol. Chem.*, 1947, **169**, 389.

TABLE IV.
ATP-ase of the Uterus Through the Estrus Cycle.
 μg Inorganic P Liberated in 15 min per mg Dry Tissue.

Stage	No. of trials	Skeletal muscle		Uterus	
		Avg	Range	Avg	Range
Estrus	2	35.5	(35-36)	125.0	(120-130)
Metestrus	9	33.7	(25-56)	93.9	(62-122)
Diestrus	3	50.0	(32-52)	107.3	(63-143)
Proestrus	2	35.5	(17-54)	106.0	(88-140)

effect. Variation in the ATP-ase activity of the uterus could not be correlated with cyclical estrus change. With the possible excep-

tion of testosterone, hormones did not appear to affect the *in vitro* systems.

16128

Production of Essential Fatty Acid Deficiency Symptoms in the Mature Rat.*

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In the course of studies on the effect of vitamin imbalance under various conditions, mature rats were maintained on restricted caloric intake of fat free diets until they lost about 50% of their original weight. In the period of recovery, during which time they were fed the same diets *ad libitum*, various symptoms including those typical of the Burr and Burr syndrome¹ were observed.

Since the original report by Burr and Burr,² several workers^{3,4,5} have observed the

typical skin symptoms of the essential fatty acid deficiency, but in all cases young animals were used. So far as we know, in no case was this deficiency syndrome reported to have been produced in adult rats.

Experimental and results. Mature male rats of the Sprague-Dawley strain averaging about 225 g have been used in these experiments. The rats were placed in individual metal cages with raised screen bottoms at the beginning of the depletion period on the fat free diets. They were weighed and examined weekly or in later experiments twice weekly.

In the first experiment 4 groups of 4 rats each were placed on the 4 diets given in Table I. In addition they all received one drop of haliver oil weekly.

For a period of 2 months each rat received 6 g of food daily. At the end of this period the amount was decreased to 5 g per day. On the 91st day one rat died in each of the groups I, II, and IV (none in the "B + supplement" group). The rats which died weighed 94, 93 and 95 g respectively. On this day all the rats were allowed their respective diets *ad libitum*. The only symptom observed by this time besides the severe emaciation

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[†] Government of India Research Fellow.

¹ Burr, George O., and Burr, M. M., *J. Biol. Chem.*, 1930, **86**, 587.

² Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345.

³ Graham, C. E., and Griffith, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 756.

⁴ Turpeinen, O., *J. Nutrition*, 1938, **15**, 351.

⁵ Hume, E. M., Nunn, L. C. A., Smedley-Maclean, I., and Smith, H. H., *Biochem. J.*, 1938, **32**, 2162.

TABLE I.
Composition of Diets.

Dietary component	I	II	III	IV
	Diet A	Diet B	Diet B + supplement	Diet C
Casein (fat free)*	30.0	30.0	30.0	30.0
Sucrose	66.0	66.0	66.0	56.0
Salt mixture IV†	4.0	4.0	4.0	4.0
Dried brewer's yeast	—	—	—	10.0
Thiamine HCl	.20 mg	per 100 g of diet 20.00 mg	20.00 mg	—
Riboflavin	.40	40.00	40.00	—
Pyridoxine HCl	.20	2.00	2.00	—
Calcium pantothenate	.30	3.00	3.00	—
Choline chloride	120.00	120.00	120.00	120.00
3-Methyl-naphthaquinone	.50	.50	.50	.50
Biotin	—	—	.02	—
Folic acid	—	—	.20	—
Inositol	—	—	50.00	—
p-Aminobenzoic acid	—	—	15.00	—
Niacin	—	—	.60	—
Corn oil	—	—	500.00	—

* Extracted for three 2-hour periods with boiling alcohol.

† Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

was slight rustiness of fur about the shoulders and neck.

On the 107th day (16 days after the start of the *ad libitum* feeding) various skin symptoms were noticed in the rats of the first 2 groups. Among these, scaly hind paws were almost uniformly present. Other symptoms which appeared among the rats, though not uniformly, were: scaly and/or annular tail; necrosis and loss of tip of tail; sloughing off of skin near base of tail and/or around the mouth; loss of hair around the mouth, eyes, over fore paws, at the base of tail and around the scrotum; thinness of hair coat over back, chest and abdomen; some edema of fore and/or hind paws and prolaps penis. The animals receiving the diet containing yeast developed scaly paws and other skin symptoms a few days later than the first 2 groups, while the group on the purified diet which was supplemented, as mentioned above (Table I), with both additional vitamins of the B complex and with 0.5% corn oil showed practically no symptoms at any time.

When these symptoms were evident, the animals in Groups I and IV were supplemented with 0.5% corn oil and those in Group II with the extra 5 vitamins included in diet III in order to see which of these factors was involved in the prevention of

the symptoms. The results of this experiment are summarized in Table II. Those animals which received additional vitamins (no corn oil) still showed scaly paws 7 weeks later, the time at which they were killed. All the rats which received the corn oil after having demonstrated the symptoms, started to show signs of recovery in 2 weeks and were completely cured within 5-6 weeks.

Along with the caloric restricted rats there was an equal number of rats on *ad libitum* regimen from the beginning of the experiment. None of these showed any symptoms at any time.

In the second experiment Diet A was used as the basal diet, and all the rats were again supplemented with one drop haliver oil weekly. The depletion period lasted 39 days, during which time the average weight dropped from 225 to 120 g. The rats were divided into two groups at this time and one group was continued on Diet A, while the other received Diet A supplemented with 0.5% corn oil. The results are summarized in Table III.

The effectiveness of corn oil in preventing development of the symptoms is obvious. The only symptoms which appeared in 5 rats of the supplemented group were slight and transient. Again the appearance of the

TABLE II.
 Condition of animals at Different Periods.

	Beginning of exper.	End of depletion	Appearance of scaly paws		Beginning of disappearance of scaly paws due to ½% corn oil	Complete clearing before exper. terminated
I—Diet A						
Days	0	91	107		120	154
Total No. rats	4	3	3		3	3
No. rats affected			3		3	3
%			100		100	100
Weight	223	118	153		200	334
II—Diet B						
Days	0	91	107		120	154
Total No. rats	4	3	3			3
No. rats affected			3			0
%			100			0
Weight	223	118	188		241	286
III—Diet B + supplement						
Days	0	91	107		120	154
Total No. rats	4	4	4		4	
No. rats affected			0			
%			0			
Weight	223	130	229		285	338
IV—Diet C						
Days	0	91	107	112	120	154
Total No. rats	4	3	3	3	3	3
No. of rats affected			0	3	3	3
%			0	100	100	100
Weight	223	125	209	238	266	317

other skin symptoms was observed in a large number of the rats on the unsupplemented diet.

Five of the rats from the unsupplemented group were given 2 drops of corn oil daily after they demonstrated the marked symptoms. Some improvement was noticed in all animals within 2 weeks, and they were all completely clear of symptoms within 3-5 weeks.

Ten rats were kept on the unsupplemented Diet A for 71 days. At the end of this period it was noticed that the scaliness of the paws had completely disappeared.

In the third experiment the animals were placed for 3 weeks on restricted amounts of our stock ration containing: ground wheat 25.00, soybean oil meal 10.00, linseed oil meal 10.00, ground yellow corn 29.00, powdered skim milk 12.00, alfalfa meal 8.00, butter 5.0, NaCl (iodized) 1.0, CaCO₃ 0.5. This was followed by 4 weeks on restricted amounts (5 g/day/rat) of fat free Diet A, supplemented by one drop of haliver oil weekly. At the time when *ad libitum* feeding was started the rats were divided into 4 groups

receiving Diet A with supplements listed in Table IV, plus one drop of haliver oil/week/rat. To guard against the possibility that the ultimate disappearance of the scaly paws of the unsupplemented animals was due to very small amounts of essential fatty acids in the haliver oil fed, in this and the following experiments carotene, calciferol (D₂) and alpha tocopherol suspended in propylene glycol or glyceryl (mono) laurate or methyl oleate were substituted for the haliver oil at the time when the scaliness of the paws appeared.

All of the 9 animals which received no essential fatty acid showed development of scaly paws within 2 weeks after the initiation of the *ad libitum* feeding. The 10 rats which received either ethyl-linoleate or corn oil did not develop the scaly paws except for a slight scaliness in 3 animals, but this disappeared in a few days. The average weights of the animals at the commencement of *ad libitum* feeding and at subsequent periods are recorded in Table IV.

It should be noted that in the 9 animals

TABLE III.
Average Weight and Number of Rats.

Supplement	Starting <i>ad lib.</i> feeding	Developing scaly paws within 17 days	Developing scaly paws within 28 days	Clearing in 1-2 weeks	Kept for 71 days	Cleared up spontaneously within 71 days
None	31 (126)*	26 (166)	31 (230)	0	10	10
0.5% corn oil	11 (129)	5 (slight) (182)	— (230)	5	—	—

* The numbers in parentheses indicate average weight.

TABLE IV.
Average Weight and Number of Rats.

Supplements	In a group starting <i>ad lib.</i>	Developing scaly paws within 2 weeks and lasting longer than 1 week	Showing scaly paws after 6 weeks	Showing complete clearance at 66 days
None	4 (114)*	4 (166)	4 (224)	4
2 drops oleic acid daily	5 (116)	5 (187)	5 (251)	5
2 drops ethyl linoleate daily	5 (117)	0 (178)	0 (268)	—
2 drops corn oil daily	5 (117)	0 (178)	0 (274)	—

* The numbers in parentheses indicate average weight.

which received no source of linoleic acid scalliness of the paws disappeared spontaneously, and within 66 days from the start of *ad libitum* feeding these animals, all of which had shown this symptom, were completely cured. The other skin lesions, when present, cleared much earlier.

This spontaneous disappearance of skin lesions within several weeks and paw scalliness within 66 days was again observed in a similar experiment carried out later with approximately the same number of animals on fat free ration A.

Discussion. Burr and Burr¹ stress the importance of scalliness of the feet as the most sensitive test of the essential fatty acid deficiency condition. Hume, Nunn, Smedley-Maclean and Smith⁵ observed dryness occurring invariably on the dorsal surface of the hind feet and front of the ankles, and therefore they finally adopted it as the most satisfactory criterion, rather than relying entirely upon weight increase which is a non-specific response. The objection to the use of skin symptoms as criterion because of their irregular appearance⁶ which is due to variation in relative humidity⁷ is obviated

in our work by the constant 50% relative humidity kept in our animal room.

Although the rats in the first experiment were maintained for a long period (3 months) on a restricted intake of fat free diets, none of the symptoms described above appeared before about 2 weeks after *ad libitum* feeding commenced, a period during which there was rapid regain of weight. In later experiments, the depletion periods on the fat free diet were considerably shorter (4-6 weeks); yet the symptoms again appeared within 2-3 weeks after the beginning of the *ad libitum* regimen.

The typical symptoms of essential fatty acid deficiency were produced heretofore only when young rats were used and generally after very long periods of fat deprivation,^{8,9} yet in our work the mature rats demonstrated the symptoms after a total period of 6-7 weeks

⁶ Evans, H. M., and Lepkovsky, S., *J. Biol. Chem.*, 1932, **96**, 587.

⁷ Brown, W. R., and Burr, G. O., *Proc. Am. Soc. Biol. Chem.*, 1936, **8**, XVI.

⁸ Hume, E. M., and Smith, H. H., *Biochem. J.*, 1931, **25**, 300.

⁹ Gregory, E., and Drumund, J. C. Z., *Vitamin-forschung*, 1932, **1**, 257.

on a fat free diet. Therefore, the technique seems to suggest itself for other types of experiments where a rapid "growth" (weight gain) or some "strain" on body functions is desired.

The ultimate disappearance of the scaly paws in these mature rats may be explained on the basis of: (1) lower demand for essential fatty acids when the rate of tissue building reaches normal maintenance level, (2) synthesis of at least a limited amount of an "essential" fatty acid within the animal itself, or (3) the combined effect of 1 and 2 in these mature animals.

Smedley-Maclean and Hume¹⁰ found that the polyunsaturated acid content of the tissue of fat starved rats fell to a very low level during the first 6 months of fat starvation. In our rats the total amounts of essential fatty acids in the emaciated bodies at the commencement of *ad libitum* feeding is probably very low. Smedley-Maclean and Nunn¹¹ concluded that in the process of growth comparatively large quantities of arachidonic acid are utilized and disappear. In the light of these findings and the observed weight gains in our rats with the beginning of the *ad libitum* feeding of the fat free diet it seems likely that they further depleted their supply of essential fatty acids when the scaliness of the paws appeared. After this

there was further weight gain in which essential fatty acids were further utilized, yet the scaly symptoms finally disappeared without any external source of these acids. Therefore, it seems likely that at least a limited amount of the essential fatty acids is synthesized in the bodies of these mature rats. This appears to be in agreement with the work of Harris¹² who used the deuterium technique and found that under appropriate conditions the rat can synthesize the essential fatty acid arachidonic acid.

Summary. 1. Mature rats were maintained on a fat free diet for relatively long periods without showing any deficiency symptoms except emaciation.

2. Severe depletion followed by *ad libitum* feeding of the fat free diet precipitated in mature rats symptoms including those typical of essential fatty acid deficiency.

3. This method is suggested for other types of experiments where a "strain" on body functions is desired.

4. Either ethyl linoleate or corn oil prevented entirely or cured rapidly the symptoms of essential fatty acid deficiency when they appeared.

5. When the rats, after depletion, were maintained on the fat free diet *ad libitum* for sufficiently long periods, spontaneous disappearance of all the symptoms was observed, for which the explanation of some synthesis of essential fatty acid is suggested.

¹⁰ Smedley-Maclean, I., and Hume, E. M., *Biochem. J.*, 1941, **35**, 990.

¹¹ Smedley-Maclean, I., and Nunn, L. C. A., *Biochem. J.*, 1940, **34**, 884.

¹² Harris, R. S., 24th Annual Report of the National Live Stock and Meat Board, 1946-47.

Effect of Carbon Dioxide and Other Gases on Electrocardiogram of the Right Ventricle.*

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It has been reported^{1,2} that the intravenous injection of carbon dioxide causes the formation of a gas bubble in the right ventricle with a concomitant lowering of the initial ventricular deflection of the electrocardiogram when this is recorded directly from the surface of the right ventricle. Further investigation has shown that this electrical phenomenon occurs not only with carbon dioxide, as was first thought^{1,2} but also with the parenteral injection of other gases.

Procedure. Mongrel dogs, averaging 6-12 kilos in weight, were anesthetized with sodium pentobarbital (approx. 35 mg/kilo) by vein. Respirations were maintained through a tracheotomy tube after the chest had been opened with a sternum-splitting incision. In addition to the standard electrocardiographic leads, direct leads were taken by placing a wick electrode without pressure on the heart muscle through a small slit in the pericardium. The various gases were injected through an exposed vein.

The electrocardiograms were taken with a Cambridge Simplitrol, using a fast speed (50 mm/sec.), a thick string casting a 3 mm shadow, and increased intensity of the light source (using a variac transformer). These three improvements made the elements of the QRS complex of the electrocardiogram much easier to read.

Results. As has already been described^{1,2} when 50-100 cc of a gas is rapidly injected into the femoral vein of a small dog (6-10 kilos), lying supine, the gas collects in the right ventricle, blocking the pulmonary conus

and artery, preventing the passage of blood into the lungs. The right heart dilates; the blood pressure falls. If the gas is carbon dioxide, which is quite soluble, it is readily absorbed by the blood in 15-17 seconds. Circulation through the lungs is resumed as the bubble is reabsorbed and the dog recovers. Other gases or mixtures of gases, such as oxygen, nitrogen, helium and air, are not so readily absorbed, and if the dog's position is not changed, death will occur in 3-5 minutes because of the complete block to the pulmonary circulation. If the gas bubble is not large, the pulmonary circulation may be re-established by turning the animal into the left lateral position.³ The gas bubble then floats up from the pulmonary conus to the apex of the right ventricle and allows blood to flow into the pulmonary artery.

As the gas bubble dilates the right ventricle an interesting electrocardiographic change takes place. The initial upward deflection of the electrocardiogram in a direct lead from the right ventricle is lowered or disappears entirely. If only a small amount of gas (10-15 cc) such as carbon dioxide is used, the R wave is merely lowered. With 50 cc or more of carbon dioxide the R wave disappears entirely (Fig. 1). At the same time the P wave increases in amplitude and the ST segment may be slightly raised, with inversion of the T wave. There is no change in the PR interval or in the width of the QRS complex. As the carbon dioxide is absorbed the R wave reappears and the electrocardiogram assumes its control appearance. Repeated injections of CO₂ produce the same sequence of events.

In fatal experiments with the less soluble

* Supported in part by RG 194, N.I.H.

¹ Durant, T., Long, J., and Oppenheimer, M. J., *Am. J. Med. Sc.*, 1947, **213**, 633.

² Durant, T., Long, J., and Oppenheimer, M. J., *Fed. Proc.*, 1947, **6**, 435.

³ Durant, T. M., Long, J., and Oppenheimer, M. J., *Am. Heart J.*, 1947, **33**, 269.

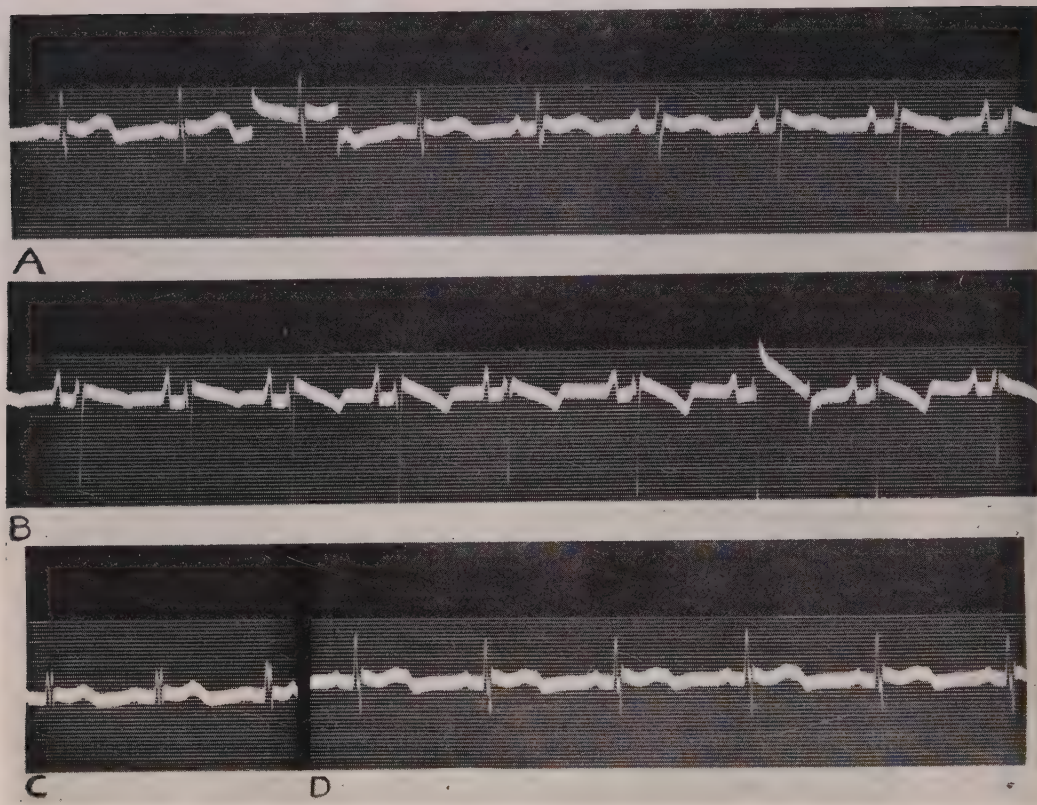


FIG. 1.

Male dog, 8.2 kg 9/16/47. A. Two control complexes and beginning of injection of CO_2 at millivolt signal (3 millivolts). B. Continuation of A with no complexes omitted. End of injection at millivolt signal. C. Between the end of B and the beginning of C 5 minutes have elapsed. D. Between C and D 5 minutes have elapsed. Anesthesia: Pentobarbital sodium, 35 mg/kg. Camera speed 50 mm/sec. Direct lead from right ventricle.

gases the R wave disappears and may not reappear. In addition the ST segment and the T wave changes become gradually more pronounced.³

The etiology of the change in the QRS complex is of interest. It is known that interposing an insulating substance such as air in hyperdistended lungs between the heart and the exploring electrode of chest leads will cause a lowering of the voltage of the R wave.^{4,5} Katz and co-workers⁶ showed that when parts of the heart surface are short-circuited by means of lead or tinfoil or in-

sulated by glass or rubber, that the voltage of the electrocardiogram is decreased. Any such etiology is ruled out in these experiments because the exploring electrode is directly on the heart muscle.

Another possible cause of the electrocardiographic change is the anoxia caused by the interruption of the blood flow through the lungs. Harris and Randall⁴ showed that the reduction of the height of the R wave in anoxic dogs with closed chests was associated with the enlargement of the thorax as a result of the hyperpnea and that there was no change in the height of the R wave in direct leads from the heart in dogs with open chests in concentrations of oxygen as low as 7%.

When animals in our laboratory were sub-

⁴ Harris, A. S., and Randall, W. C., *Am. J. Physiol.*, 1944, **142**, 452.

⁵ Lapin, A. W., *Am. Heart J.*, 1947, **33**, 747.

⁶ Katz, L. and Korey, H., *Am. J. Physiol.*, 1935, **111**, 83.

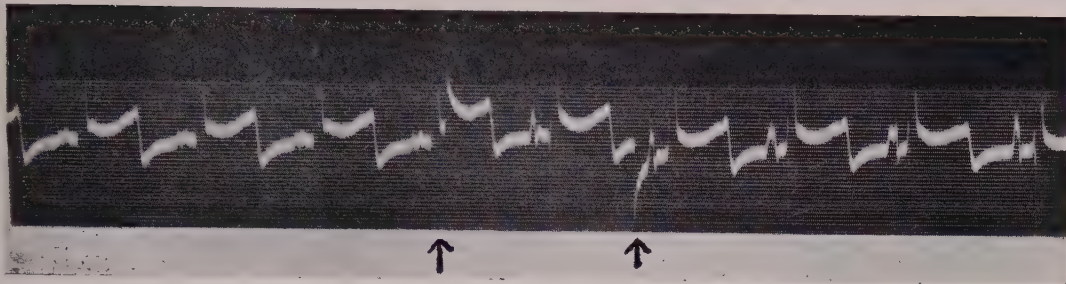


FIG. 2.

Male dog, 8.0 kg 9/30/47. Three control complexes and beginning of mineral oil injection at millivolt signal (3 millivolts). Anesthesia: Pentobarbital sodium, 35 mg/kg. Camera speed, 50 mm/sec. Direct lead from right ventricle. R_1 and R_2 are present in the control complexes. R_1 alone is diminished by the injection of mineral oil. The two arrows indicate the duration of the millivolt signal.

jected to asphyxia by cutting off the artificial respiration, the right heart dilated but there was no lowering of R.

Sudden dilatation of the right ventricle by means of the rapid injection of 100 cc of blood into the jugular vein or by compressing the pulmonary artery was found to be ineffective in changing the amplitude of the R wave.

An important possibility to consider is that the electrocardiographic changes are due to the replacement of blood in the right ventricular cavity by a dielectric. It was found that when mineral oil, a non-conducting liquid, was injected rapidly in sufficient quantities to completely replace the blood in the right ventricle (50 to 100 cc in a 10-kilo dog) the same phenomenon occurred, *i.e.* there was a marked lowering or disappearance of the R wave (Fig. 2). In each case a post-mortem check of the contents of the ventricle was made. In one experiment, wherein only 50 cc of oil was injected in a 12-kilo dog, the R wave was unchanged, and at post-mortem

the right ventricle was found to contain some oil mixed with a large amount of blood, hence complete replacement by a dielectric had not occurred.

The injection of carbon dioxide into the left ventricular cavity in several experiments resulted in the production of multiple extrasystoles, making it impossible to evaluate its effect on the R wave in leads from the left ventricle. Moreover, we have been unable to adequately fill the left ventricle with mineral oil so as to study the effects of this dielectric on left ventricular leads. Furthermore, these experiments are complicated by the effects of coronary artery filling by the injected substance.

Conclusion. The rapid intravenous injection of gases or mineral oil in sufficient quantities to replace blood in the right ventricle of dogs will cause the reduction or loss of the initial ventricular deflection of the electrocardiogram in direct leads from the epicardial surface of the right ventricle.

Methionine Excretion. Effect of Diet and Methionine Ingestion in Normal Subjects.

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A number of reports¹ in the literature have indicated the outstandingly important role that methionine may play in nitrogen metabolism. Its utilization has been shown to involve not only the intact amino acid but also the sulfur and labile methyl groups in the formation of other essential substances. The varied uses of methionine have been shown by its lipotropic activity and protein sparing action in animals on very low protein diets, and by its role in the detoxification of a variety of organic compounds.²

The recently emphasized physiological importance of methionine, along with our earlier studies³ on animals with fatty livers produced by diets deficient in this amino acid, suggested an investigation of methionine excretion as a new approach to the study of methionine metabolism and as a possible indication of its utilization.⁴ Changes in urinary methionine excretion might be caused by increased requirements for this amino acid for essential body proteins. Also increased requirements, which could not be met from available methionine, might be reflected by a decreased excretory response to a test supplement.

Information about the excretion of methionine by normal individuals is scanty. Cleland,⁵ in her study of methionine excretion in cystinuria, obtained normal values of the same order as those found by Albanese and coworkers,^{4,6} and they both reported an im-

mediate rise in its excretion after ingestion of the free amino acid. If an increase in the protein intake should cause a rise in the excretion of methionine as in the cystinuric,⁵ then the effect of fasting and of various dietary regimes, such as low protein, higher protein, and high fat diets, upon its urinary loss should be considered in excretion studies.

Methods. The subjects used in this study were 80 male medical students. The urinary samples for methionine analysis were collected over the 6-hour period, 7 a. m. to 1 p. m., and for the subsequent 18 hours when 24-hour collections were desired. The 6-hour collections were included as a more convenient period of measurement since Albanese⁴ has shown a return to the normal level of excretion 3 hours after an intake of 1.5 g of methionine, with equal utilization of the natural and racemic forms. Usually on the following day at 7 a. m., the subjects, fed or fasted, were given 1.0 or 1.5 g of *dl*-methionine orally. The urine collections were started at that time and completed at the end of the same periods used for the controls.

In a second experiment, low protein, higher protein, and high fat diets were followed for 4 days by selecting food from lists of items suitable for such diets. Urine collections were made on the last day of the special diets. Besides methionine, total nitrogen and pH values were determined on these urine samples to indicate how well the diets had been followed. The subjects on high fat diet ingested 20% cream which contains about 2.5 times more ketogenic than antiketogenic substances. This accounts for the fair intake of methionine and only traces of acetone in the urine. They

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¹ Lugg, J. W. H., *Ann. Rev. Biochem., Stanford University*, 1945, **14**, 271.

² Miller, L. L., *J. Biol. Chem.*, 1944, **152**, 603. Also references 1 and 5-7.

³ Treadwell, C. R., Tidwell, H. C., and Gast, J. H., *J. Biol. Chem.*, 1944, **156**, 237.

⁴ Albanese, A. A., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 175.

⁵ Cleland, J. B., *Australian J. Exp. Biol. and Med. Science*, 1945, **23**, 273.

⁶ Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, 1944, **156**, 293.

were given a supplement of 1.5 g of methionine and only the 6-hour collections were examined. Separate normal excretion values were obtained on these subjects at this time.

The second experiment was repeated in part on a similar group of students one year later. In addition, 24-hour urine collections, after fasting and a high fat diet, were obtained for examination. Both of these groups developed a marked ketonuria.

In all these studies, total nitrogen excretion values were used to calculate the protein intake of the subjects on the various diets, after allowing 3 days on the diet for stabilizing the plane of nitrogen metabolism. Methionine intakes were estimated by assuming that the protein of the diet contained an average of 3% methionine.⁷ The urinary methionine was determined according to the method of Albanese *et al.*⁴ Quantitative recovery of methionine added to the urine by us indicates the suitability of the method for the estimation of methionine in urine. However, it must be remembered that the method will respond to any other unknown terminal thio ether or thiocarbonyl group containing substances of the urine. The data were analyzed according to the *t* method of Fisher⁸ and only those results showing a *P* value of 0.01 or less were considered significant. The standard error of the mean for each value is given with the data.

Results. Our results on the daily urinary excretion of methionine by the adult male compare favorably with those of Albanese *et al.*⁴ and Cleland.⁵ The former group found an excretion of 247 to 494 mg of methionine per day in a series of 7 observations. Using 80 different subjects, we obtained an average daily excretion of 318 ± 9 mg (range 199 to 518 mg) or about 4.5 mg per kg body weight.

The methionine excretion during 6- and 24-hour periods and following the ingestion of a 1.0 or 1.5 g supplement is shown in

Table I. The latter supplement was approximately one-half of Block's estimate⁹ of the average daily intake of methionine. An increased amount of methionine was excreted after the one g test supplement and definitely more after the 1.5 g. However, the percentage of the supplement excreted was the same in both cases, approximately 9% in 6 hours and 15% in 24 hours respectively. An overnight fast caused only a slight change in the methionine excreted, either in the absolute amount of the amino acid, or in the per cent of the test supplement given. Thus, if excretion be taken as a measure, the major part of the free amino acid was utilized (stored or metabolized) even when the intake was markedly increased by the additional methionine ingested.

The remainder of this study was concerned with the effect of fasting and of various diets upon the daily methionine excretion in the normal subject. Three types of diet were ingested—low protein, higher protein, and high fat diets. The total nitrogen of the urines of subjects on these diets served as a check upon the protein intake. Averages of 6.5, 17.7, and 9.3 g of total urinary nitrogen per day, respectively, were obtained in the second experiment. The pH of the urines, the methionine intakes and amounts excreted are included in Table II. Similar data for the third experiment are shown in Table III.

There was no significant difference in the final 24-hour urinary methionine excretions by the subjects on the low and higher protein diets for a period of 4 days. This was true in both experiments on humans (Table II and III), and the observation has been confirmed with rats.¹⁰ These results are supported by the relative constancy of the neutral sulfur excretion.¹¹ Thus marked variations in the protein content of the diet,[†] did not affect the daily urinary loss of methionine in these

⁹ Block, R. J., *Yale J. Biol. Med.*, 1943, **15**, 723.

¹⁰ Tidwell, H. C., unpublished data.

¹¹ Deuel, H. J., Jr., Sandiford, I., Sandiford, K., and Boothby, W. M., *J. Biol. Chem.*, 1928, **76**, 391.

[†] Since completion of these studies, a similar observation has been reported by Johnson, R. M., Deuel, H. J., Jr., Morehouse, M. G., and Mehl, J. W., *J. Nutrition*, 1947, **33**, 371.

⁷ Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Natural Foods*, C. C. Thomas, Springfield, Ill., 1944.

⁸ Fisher, R. A., *Statistical Methods for Research Workers*, 7th ed., Oliver and Boyd, Edinburgh, 1938.

TABLE I.
Effect of Methionine Ingestion upon Its Excretion.

The fed subjects ingested their normal diets at the usual hours while the fasted subjects had no food for 18 hours including the 6 hours of the urine collection. The normal diets contained approximately 2 g methionine per day.

No. of subjects	Nutritional state	Methionine supplement, g	Methionine excreted			
			6 hr		24 hr	
			total, mg	per kg, mg	total, mg	per kg, mg
11	Fed	0.0	99	1.41	406	5.79
			± 5	± 0.07	± 13	± 0.02
11	"	1.0	185	2.65	550	7.85
			± 11	± 0.16	± 18	± 0.28
15	"	0.0	98	1.37	415*	5.73*
			± 5	± 0.20	± 32	± 0.46
15	"	1.5	229	3.16	665*	9.34*
			± 9	± 0.15	± 41	± 0.46
11	Fasted	0.0	83	1.15		
			± 5	± 0.07		
11	"	1.5	206	2.85		
			± 7	± 0.18		

* Includes 8 subjects.

TABLE II.
Effect of Diet on Urinary Methionine Excretion.

Exp. 2. Subjects ingested the special diets for 4 days. Determinations were made on samples collected during the 6 or 24 hours of the last day. Daily methionine intake was estimated by assuming that the protein of the diet contained 3% methionine.⁷

No. of subjects	Dietary regime	Urine pH	Calculated methionine intake, g/day	Methionine excreted	
				total, mg	per kg, mg
12	Low protein	6.2	1.22	267	3.75
		± 0.2	± 0.11	± 15	± 0.19
9	Higher protein	5.6	3.32	252	3.52
		± 0.1	± 0.06	± 19	± 0.24
28	Average			257	3.62
				± 11	± 0.13
9	Normal	6.5	2.10	85†	1.24†
		± 0.3		± 5	± 0.14
9	" *		2.10	223†	3.11†
			± 0.15	± 11	± 0.15
11	High fat*	5.0	1.74	154†	2.25†
		± 0.1	± 0.13	± 9	± 0.13

* After a 1.5 g test supplement of methionine.

† Determinations made on 6-hr collections.

subjects. The increased methionine excretion after the ingestion of the free amino acid as compared with that after its ingestion in the form of protein might be explained by an increased rate of absorption from the gastrointestinal tract. Fratzer¹² found that the oral administration of hydrolyzed protein produced a greater increase in the blood amino acid level than did the ingested intact protein,

and that methionine caused higher levels than did some of the other amino acids. The higher blood levels might account for the increased methionine excretion after the ingestion of the free amino acid.

Although a marked change in the protein intake was without effect on the urinary methionine excretion, a 3-day fast or high fat diet was associated with a significantly decreased excretion as compared with that on

¹² Fratzer, F. H., *J. Biol. Chem.*, 1944, **153**, 237.

TABLE III.
Effect of Diet on Urinary Methionine Excretion.

Exp. 3. Determinations were made on 24-hour samples of the fourth day on these dietary regimes. Daily methionine intake calculated as in Table II.

No. of subjects	Dietary regime	Urine pH	Total N excreted, g/day	Calculated methionine intake, g/day	Methionine excreted	
					total, mg	per kg, mg
13	Low protein	6.6	7.15	1.34	298	4.18
		± 0.1	± 0.34	± 0.07	± 14	± 0.20
12	Higher protein	6.0	17.74	3.33	290	4.21
		± 0.1	± 1.29	± 0.24	± 12	± 0.26
12	High fat	5.7	11.88	2.23	252	3.48
		± 0.2	± 0.97	± 0.18	± 30	± 0.43
9	Fasting	5.1	11.95	0	269	3.42
		± 0.0	± 0.74		± 26	± 0.39

the low and higher protein diets used. Also, there was a marked decrease in the urinary methionine after a test supplement was given the subjects on the high fat diet as compared with the normal control values as shown in Table II. Circumstances prevented 6-hour high fat control values on these subjects, but a decreased loss of methionine was found in a 24-hour test in a similar group in the third experiment. Hence a lowered base level of excretion, and a possible slower rate of absorption, might account for a part of the decreased excretion of the supplement in these subjects. However, it is evident that a very high fat intake or fasting, accompanied by a ketonuria, was associated with a decrease in methionine excretion. Increased needs for methionine for lipotropic requirements at times of excessive fat metabolism, owing to a very high fat intake or the excessive use of body fat during fasting, might account for the decreased excretion in these subjects. In this case greater body needs appear to be followed by a decreased excretory response.

The excretion of less methionine by the subjects on the special diets as compared with that of the others in this study was associated with smaller urine volumes during warmer weather. The urines for the dietary studies were collected around May 30 when the average temperature was above 80°F, while the other values were obtained during February and March with temperatures averaging near 60°F. Lack of other known variables than the temperature suggested possible losses of methionine through channels other than

the kidneys. Nevertheless, we were unable to correlate the daily urine volumes and the urinary methionine excretion in the various experiments.

Another interesting fact was noted when the per cent retentions of methionine on the different protein intakes in the last two experiments were compared. Values of 78% retention on the low, and 92% on the higher protein diets were almost identical in the last two experiments. This decreased loss in the latter case suggests an increased utilization of methionine with the higher protein intakes.

Summary. Observations on a series of 80 normal male subjects, on a normal control diet, indicated an average daily urinary excretion of approximately 318 ± 9 mg of methionine. The increase in methionine excretion during the 6- and 24-hour periods following the ingestion of a 1.0 or 1.5 g supplement of methionine in addition to that of the usual protein intake only amounted to approximately 9 and 15% respectively of the intake of the free amino acid.

The ingestion for 4 days of diets of varying protein content showed no change in the daily urinary methionine excretion as a result of such variation. A decrease in the methionine normally excreted occurred when the subjects were on a high fat diet or when, on a 6-hour test, they were given a similar test supplement of methionine. Increased lipotropic requirements might account for the decrease in the loss of methionine after a 3-day fast or high fat intake.

An Acoustical Indicator of the Systolic End Point in Rat Blood Pressure Determinations.*

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An electrical device for determining the systolic end point of rat blood pressures has been described by Skeggs and Leonards.¹ It consists of a small carbon telephone transmitter button placed in series with an ammeter in a low voltage wheatstone bridge circuit. Pressure on the button decreases its resistance resulting in deflection of an ammeter needle. Pressure is transmitted from the tail to the button by means of a simple lever system. Engorgement of the tail vessels and increase of size with pressure on the button occur at the systolic blood pressure level following release of occlusion of the base of the tail by an air cuff. At the moment of decrease in electrical resistance and sweep of the ammeter needle, blood pressure is read from a manometer in the air cuff system.

This electrical end point device of Skeggs and Leonards requires alternate visual observation of an ammeter needle and of a sphygmomanometer. The modification to be described was devised to facilitate and improve accuracy of readings by permitting simultaneous visual and auditory observation. A set of headphones and an amplifier circuit was substituted for the ammeter. Decrease of resistance within the carbon button at the end point is heard as a change of tone permitting undivided visual attention to the sphygmomanometer.

Acoustical Indicator. The circuit for the acoustical indicator is illustrated in Fig. 1. A sensitive carbon button similar to that of the Skeggs and Leonards apparatus is placed in the grid circuit of a 1LE3 triode amplifier tube. The neon lamp employed is of low wattage ($\frac{1}{4}$ watt) and has no internal resistor. The fixed condenser has a capacity of

0.002 Mfd. A variable resistor of 5,000 ohms is placed in series with the carbon button and another variable resistor of 7,500 ohms is put in series with the cathode and the neon lamp. A fixed carbon resistor of 270,000 ohms is required in series with the plate of the triode tube. The headphones have a resistance of 3,000 ohms.

The circuit operates on direct current from batteries. A switch is provided to disconnect all batteries except the 120 v power supply when the apparatus is not in use. This circuit produces a rapidly interrupted current audible in the headphones as a distinct tone. The tone can be varied to a higher or lower pitch by changing the two variable resistors, permitting adjustment for maximum change of pitch when engorgement of the vessels of the rat's tail produces pressure on the carbon button.

Method of Use. The technique of taking rat blood pressures with the acoustical indicator is similar to that used when employing the ammeter circuit. The rat tail is adjusted in the carbon button holder and then the acoustical indicator is switched on. The pressure in the air cuff is elevated above the expected blood pressure and then slowly released. The tone in the headphones rises during inflation of the cuff and decreases with release of the cuff pressure until the systolic end point is reached when the pitch again increases. The end point is indicated by a smooth, rapid increase in pitch which, after attaining its maximum height, will be maintained until the cuff pressure is lowered 20 to 70 mm Hg below the systolic pressure. Artifacts produced by movements of the tail are distinguishable as rapid, transient changes in pitch.

Unfortunately it has not been possible to obtain blood pressure readings without producing vasodilatation of the tail by preparing the rat. The conventional method of obtaining

* This research was supported by a grant from the Ciba Pharmaceutical Products, Inc., Summit, N. J.

¹ Skeggs, L. T. and Leonards, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 294.

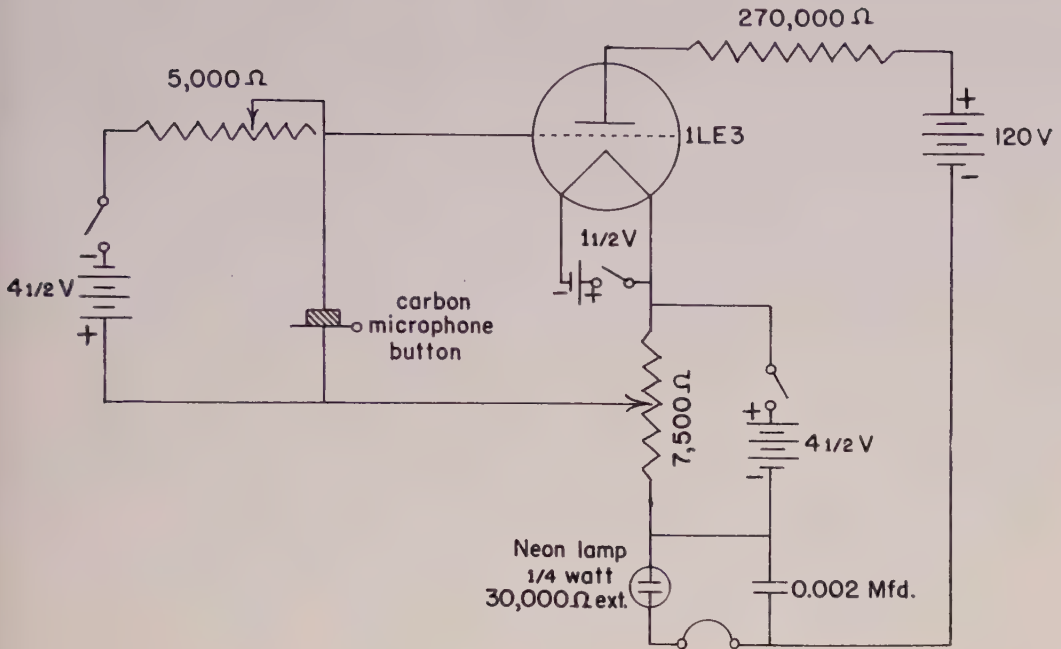


Fig. 1

vasodilatation is heating of the entire rat or of the tail alone. Williams, Harrison, and Grollman² have reported heating of the entire rat, exposing it to 40-42°C for 10 minutes before obtaining readings. They used the plethysmograph technique. Sobin³ has reported equal success after heating the tail alone. Proskauer, Neumann, and Graef⁴ have reported that heating elevates rat blood pressure. In our experience heating of the entire animal at 40-42°C for 10 minutes increased the rectal temperature on the average 3.3°. Heating the tail alone at 42-45°C increased the rectal temperature on the average 1.9°C. However, heating either the entire animal or the tail alone produced comparable readings of systolic blood pressure.

Another method of obtaining readings is the use of a peripheral vasodilating agent. 2-Benzyl-4, 5-Imidazoline HCl (Priscoll) dilates the tail vessels of the rat and permits determination of systolic blood pressure without increase of temperature of the rat. Ad-

ministration of 3-4 mg/kg Priscoll intramuscularly permitted blood pressure readings after 45 to 60 minutes which could be repeated at will during a period up to 9 or 10 hours after the drug.

Either heat to the entire rat or Priscoll was used for the experiments below comparing 3 methods for blood pressure determinations, Grollman (plethysmographic), Skeggs and Leonards, and the acoustical modification.

Experimental Evaluation. Readings of the rat systolic blood pressure were compared using the Grollman plethysmograph technique, the ammeter circuit, and the acoustical indicator. (Table I). Simultaneous readings were obtained with the plethysmograph and one or the other electrical end point devices. Readings were repeated alternating the electrical methods thereby comparing all 3 methods in 6 to 7 minutes. Plethysmographic readings were consistently lower than obtained with either electrical device. The average difference in blood pressure was 3-5 mm Hg with heated rats and 9-10 mm Hg with unheated Priscoll treated rats. Both electrical end point devices would often give blood pressure readings following Priscoll preparation when they were unobtainable with the

² Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

³ Sobin, S. S., *Am. J. Physiol.*, 1946, **146**, 179.

⁴ Proskauer, G. G., Neumann, D., and Graef, I., *Am. J. Physiol.*, 1945, **143**, 290.

TABLE I.

Comparison of Systolic Blood Pressure Readings by 3 Methods in Heated Normal Rats and in Priscol Treated Rats Without Heating.

Rat	Rats heated 10 min. at 40°-41°C			Priscol treated		
	Plethysmograph	Ammeter	Headphones	Plethysmograph	Ammeter	Headphones
1	108	112		* 90		104
	112	110		90		94
	112	114		88		92
	104		114	90	110	
	106		108	100	106	
	110		116	98	102	
2	98		102	* 100	112	
	98		98	98	104	
	96		96	90	108	
	90	90		96		102
	90	90		94		102
	88	88		90		98
3	116		122	† 70		88
	114		118	78		92
	114		120	84		104
	118	122		90	94	
	116	116		86	96	
	112	116		82	90	
4	84	88		† 80	94	
	82	84		90	90	
	84	84		86	92	
	78		82	82		96
	80		84	84		96
	78		82	80		94

* 3 mg/kg Priscol I.M. † 4 mg/kg Priscol I.M.

plethysmograph. This greater sensitivity was observed during the periods of minimum vasodilatation before and after the maximum effect. Frequently readings taken by the ammeter circuit and the acoustical indicator coincided. However, the acoustical indicator gave readings that averaged 1.25 mm Hg above those of the ammeter circuit when a series of 100 readings by each device was compared.

Conclusion. The acoustical modification of the Skeggs and Leonards apparatus described

facilitates determination of the blood pressure of rats but can be used only after vasodilatation produced by heat or drugs. Priscol produces vasodilatation sufficient for repeated blood pressure determination during a 9- or 10-hour period.

We wish to express appreciation to A. A. Foster for directing our attention to the electrical circuit herein described and to L. T. Skeggs and J. R. Leonards for one of their electrical end point devices.

16132

Relaxin Content of Blood, Urine and Other Tissues of Pregnant and Postpartum Guinea Pigs.*

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Relaxin was first detected in the blood of pregnant rabbits and guinea pigs (Hisaw¹)

* Aided in part by a grant from the Donner Foundation to Prof. Frederick L. Hisaw.

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and was subsequently found in the blood of pregnant sows, dogs, cats, mares and women (Hisaw;² Brouha and Simonnet;³ Pommerenke;⁴ Abramson, Hurwitt and Lesnick⁵). Separation of the pubic bones which occurs

¹ Hisaw, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1926, **23**, 661.

during the normal gestation in the guinea pig has been described by a number of investigators and this reaction, as induced experimentally by relaxin, has been used extensively in the assay of this hormone. Nevertheless, except for the rabbit (Marder and Money⁶) no attempt has been made to determine the amount of relaxin present throughout the various stages of pregnancy. Therefore, it was felt desirable to study quantitatively the concentration of relaxin in the blood, urine, uterus and placenta of guinea pigs at different times of pregnancy and after delivery of the young.

Methods. Adult female guinea pigs weighing from 600 to 800 g were mated and pregnancy dated from the morning on which the presence of vaginal plugs was noted. The average length of pregnancy was considered to be 68 days (Young and Blandau⁷). Blood was obtained from the animals by cardiac puncture at definite intervals after mating and the blood serum from a group of guinea pigs of a similar length of pregnancy pooled in order to obtain a sufficient quantity to carry out relaxin determinations. Urine samples were secured from a number of guinea pigs for a 24-hour period prior to obtaining the blood and dialyzed against running tap water. Placentæ and uteri were removed from animals on the 56th and 63rd day of pregnancy, ground up and extracted twice with 3% HCl. The extracts were kept in the cold at a pH of approximately 0.5 and prior to injection were brought to pH 7 with 10% NaOH.

The assays for relaxin content were performed according to the method of Abramo-

witz *et al.*⁸ except that the guinea pigs were pretreated with one μ g of estradiol daily for 3 days. Each sample was tested at several dilutions in order to obtain a concentration that gave a 67% response. The guinea pigs used for the relaxin determinations were part of a large colony maintained solely for assay purposes. In addition to the animals employed in the assay a total of 80 guinea pigs were used in this investigation, 57 for the study of pregnancy and 23 following parturition. The relaxin content of the blood serum was determined at 15, 21, 28, 35, 42, 49, 56, and 63 days after mating and at 2, 6, 24, and 48 hours postpartum. Twenty-four-hour samples of urine were obtained 42, 56, and 63 days after mating and the uterine and placental tissues were removed at 56 and 63 days of pregnancy.

Results. Relaxin assays on the 15th day of pregnancy were negative at a level of 10 ml of serum. However, a positive response was obtained by the 21st day indicating approximately 0.25 G.P. units per ml. (Fig. 1). Between the 21st day and the 28th day the concentration of relaxin was doubled, reaching a maximum value of 0.5 G.P. units per ml of serum. This level was maintained for 4 weeks and was followed by a drop to 0.33 G.P. units per ml by the 63rd day of pregnancy. Shortly after parturition a second and more precipitous decrease took place so that in the 48-hour postpartum guinea pig relaxin was no longer detectable in 10 ml of blood serum.

The urine showed an activity of 0.5 G.P. units of relaxin per ml at 42 and 56 days of pregnancy, and approximately 0.25 G.P. units per ml of urine on the 63rd day. It is of interest to note that the drop in the relaxin concentration of the urine occurred at the same time as that in the blood serum. The total volume of urine for a 24-hour period varied from 10 to 20 ml so that the guinea pigs excreted in their urine from 5 to 10 G.P. units of relaxin daily between the 28th and 56th days of pregnancy.

Approximately 10 G.P. units of relaxin per gram of fresh tissue was found in the uterus of the guinea pigs on the 56th day of pregnancy whereas the placenta showed a

² Hisaw, F. L., *Physiol. Zool.*, 1929, **2**, 59.

³ Brouha, L., and Simonnet, H., *Compt. rend. Soc. de biol.*, 1928, **99**, 1769.

⁴ Pommerenke, W. T., *Am. J. Obst. and Gynecol.*, 1934, **27**, 708.

⁵ Abramson, D., Hurwitt, D., and Lesnick, G., *Surg. Gynec. and Obst.*, 1937, **65**, 335.

⁶ Marder, S. N., and Money, W. L., *Endocrinol.*, 1944, **34**, 115.

⁷ Young, W. C., and Blandau, R. J., *Science*, 1936, **84**, 270.

⁸ Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmage, R. V. N., Kleinholz, L. H., and Hisaw, F. L., *Endocrinol.*, 1944, **34**, 103.

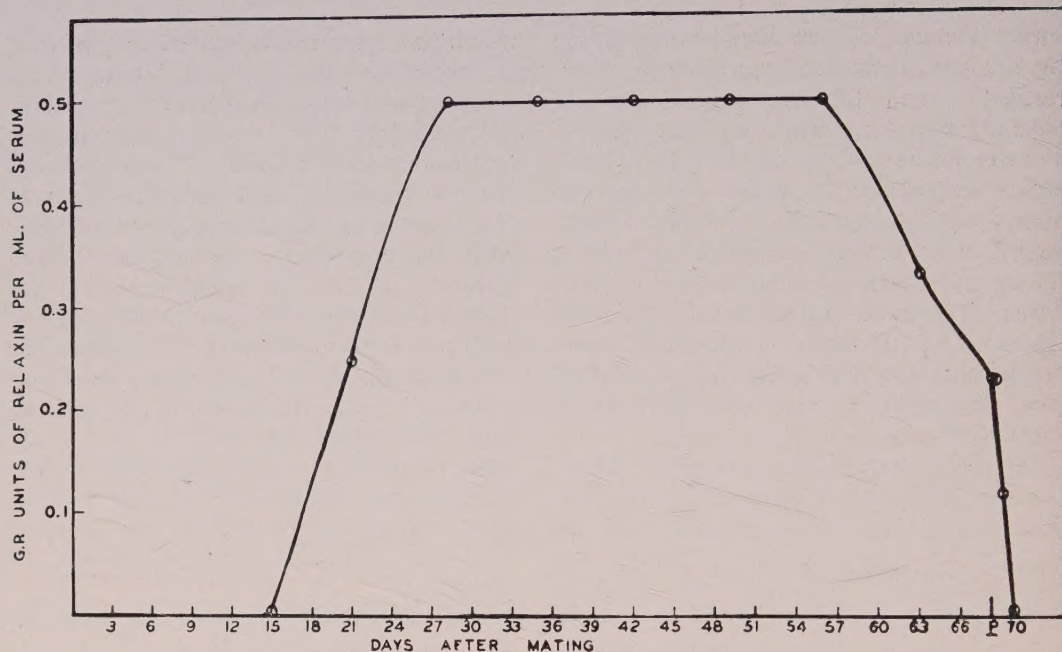


FIG. 1.

The relaxin content of the blood serum of pregnant and postpartum guinea pigs. Parturition (P) occurred 68 days after mating.

relaxin content of 5 G.P. units per gram. On the 63rd day of pregnancy the uterus still contained 10 G.P. units of relaxin per gram of fresh tissue while the placenta had only 2.5 G.P. units per gram.

Discussion. A series of 20 guinea pigs were palpated daily starting with the first day of pregnancy in order to determine the time at which relaxation of the symphysis pubis normally occurred. Relaxation was first detected on about the 20th day (range, 18 to 25 days), which compared favorably with the time at which relaxin first appears in the blood of the pregnant animals (Fig. 1.) It should be further pointed out that these two events may also be correlated with the time during which the placenta undergoes its most rapid development (Marshall⁹). There is a similar correlation in the rabbit between the development of the placenta and the appearance of relaxin in the blood (Marder and Money⁶). The maximum concentration of relaxin found in the blood of the pregnant guinea pig was 0.5 G.P. units per ml of serum which is one-twentieth the concentration

found in the pregnant rabbit.

The fall in the relaxin content of the blood serum of the guinea pig previous to parturition is significantly different from the situation in the pregnant rabbit. In the rabbit the relaxin concentration is maintained at a plateau until parturition, at which time a precipitous drop occurs (Marder and Money⁶). However, there is some evidence which indicates that the situation in the pregnant woman may be similar to that in the guinea pig. It has been found that the amount of relaxin in the blood serum of pregnant women is considerably greater during the first half of pregnancy than during the 8th month (Pommerenke,⁴ Abramson, Hurwitt, and Lesnick⁵). Thus, it would appear that in both the guinea pig and woman, unlike the rabbit, changes take place prior to parturition resulting in a decreased production of relaxin.

From the results obtained on the relaxin content of the uterus and placenta of the guinea pig at 56 and 63 days of pregnancy it would appear that the drop in the relaxin concentration of the blood serum may be due to a decreased output by the placenta. However it is not possible to account for this

⁹ Marshall, F. H. A., 1922, *The Physiology of Reproduction*. Longmans, Green and Co., London.

with certainty and the problem is further complicated by the fact that a species difference may exist with regard to the part of the female reproductive tract that may serve as a source for relaxin.

Summary. Relaxin is found in the blood of guinea pigs on about the 21st day of pregnancy, at which time relaxation of the symphysis pubis may first be detected by palpation. The concentration of relaxin reaches a peak of 0.5 G.P. units per ml of blood serum on the 28th day and is maintained until the 63rd day when the concentration falls to 0.33 G.P. units per ml. Immediately after

parturition a precipitous decline of the relaxin content of the blood serum takes place. The urine shows a concentration of 0.5 G.P. units of relaxin per ml at 42 and 56 days of pregnancy with a drop to 0.25 G.P. units by the 63rd day. The uterus contains about 10 G.P. units per gram of tissue on the 56th and 63rd day of pregnancy whereas the placenta shows 5 G.P. units per gram of tissue on the 56th day and 2.5 G.P. units on the 63rd day. It seems probable that the drop in serum relaxin in the latter part of pregnancy may be due to a decreased output from the placenta.

16133 P

Enhancement of Effect of Chorionic Gonadotrophin on Ovarian Hyperemia in the Rat by Pituitary Extract.

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The earliest demonstrable effect of chorionic gonadotrophin on the ovary of rats is the appearance of hyperemia.¹ This reaction has been used with varying degrees of success for the rapid diagnosis of pregnancy. Attempts to enhance the effect of chorionic gonadotrophin on ovarian hyperemia led to our present use of a pituitary extract for this purpose. This was based on the well-known synergistic effect of certain anterior pituitary extracts in augmenting the ovarian response when used in conjunction with chorionic gonadotrophin.² The effective component of such pituitary extracts is not known. The earlier claims³ that such "synergists" represent a distinct gonadotrophin no longer seems likely in view of later work⁴ indicating that similar effects can be obtained with follicle-stimulating gonadotrophins (F.S.H.). The pituitary synergist[†] employed in these studies is reported to be chiefly F.S.H., with traces of luteinizing principle.⁵ Prolactin was not demonstrable.

Methods and Results. The 28- to 32-day-old, 50 to 60 g immature albino female rats used were of a mixed strain of unknown identity. Chorionic gonadotrophin, the pitui-

- 2 a. Evans, H. M., Meyer, K., and Simpson, M. E., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 845.
- b. Evans, H. M., Meyer, K., and Simpson, M. E., *Am. J. Physiol.*, 1932, **100**, 141.
- c. Fevold, H. L., Hisaw, F. L., Hellbaum, A. A., and Hertz, R., *Am. J. Physiol.*, 1933, **104**, 710.
- d. Fevold, H. L., Hisaw, F. L., and Hertz, R., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 914.
- e. Leonard, S. L., *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 403.
- f. Leonard, S. L., and Smith, P. E., *Am. J. Physiol.*, 1934, **108**, 22.
- g. Smith, P. E., and Leonard, S. L., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 1248.
- h. Collip, J. B., Selye, H., Anderson, E. M., Thomson, D. L., *J.A.M.A.*, 1933, **101**, 1553.
- 3 a. Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, **57**, 897.
- b. Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, **58**, 545.
- c. Evans, H. M., Pencharz, R. I., and Simpson, M. E., *Endocrinology*, 1934, **18**, 601, 607.

* Schering Fellow in Gynecologic and Obstetric Endocrinology.

¹ Zondek, B., and Sulman, F., *Vitamins and Hormones*, 1945, vol. 3, p. 328, Academic Press, N. Y.

TABLE I.
Hyperemia Response with Varying Dosages of Chorionic Gonadotrophin and Synergist.

		Dose of synergist (units) and response											
Reaction time	Dose Chorionic Gonad. (I.U.)	None		0.044		0.022		0.011		0.0044		0.0022	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
2 hr	None	—	—	0	18	0	9	0	5	0	5	0	4
	0.60	19	6	4	0	8	0	5	0	5	0	4	2
	0.55	4	9	4	0	3	0	1	3	1	3	—	—
	0.50	0	11	5	0	3	0	0	3	—	—	—	—
	0.45	0	5	5	0	3	0	0	4	—	—	—	—
	0.40	0	5	3	0	3	0	1	2	—	—	—	—
	0.30	0	4	3	0	3	0	0	5	—	—	—	—
	0.20	0	4	0	3	0	3	—	—	—	—	—	—
	0.10	0	4	0	3	0	3	—	—	—	—	—	—
1 hr 15 min	None	—	—	0	4	—	—	—	—	—	—	—	—
	0.65	5	3	6	0	—	—	—	—	—	—	—	—
	0.60	4	5	5	0	—	—	—	—	—	—	—	—
1 hr	None	—	—	0	4	—	—	—	—	—	—	—	—
	0.70	1	7	3	0	—	—	—	—	—	—	—	—

tary synergist, or their combination was injected intraperitoneally into a total of 307 rats. The rats were asphyxiated with ether and autopsied after one, 1¼ or 2 hours. The reaction was considered positive if one or both ovaries were light to dark crimson, and negative if both were pale or pinkish. The details of the technic used have been described by Kupperman and Greenblatt.⁶

The results are given in Table I. There were no positive reactions with the synergist alone. Chorionic gonadotrophin in a dosage of 0.6 I.U. produced positive reactions in 76% of 25 rats. The percentage of positive results could not be increased by raising the dosage higher. When 0.0044 or more units of

synergist were added to this minimal effective amount[‡] of gonadotrophin, strong positive reactions were present in 100% of 22 rats. With subminimal doses of chorionic gonadotrophin alone, positive reactions began to appear only after the injection of 0.55 I.U. or more. Strong positive reactions were noted in all rats injected with 0.30 to 0.55 I.U. of chorionic gonadotrophin if 0.022 or more units of synergist were added. The combinations of 0.2 I.U. or less of chorionic gonadotrophin and the maximum amount of synergist used, 0.044 units, did not produce positive reactions.

At 1¼ hours the percentage of positive reactions with the subminimal effective amount of chorionic gonadotrophin (0.65 I.U.) was increased from 62 to 100 with the addition of 0.044 units of the synergist, while the percentage of positive reactions with 0.60 I.U. was raised from 44 to 100. It is notable that though the minimal dosage of chorionic gonadotrophin at one hour was 3.0 I.U., positive reactions with 0.70 I.U. were increased from 15 to 100% with the addition of 0.044 units of the pituitary synergist.

The enhancement of the rat ovary hyperemia effect by this pituitary extract has been used to advantage by us in testing urines for the presence of pregnancy.

⁴ a. Evans, H. M., Simpson, M. E., Tolksdorf, S., and Jensen, H., *Endocrinology*, 1939, **25**, 529.

b. Jensen, H., Simpson, M. E., and Tolksdorf, S., *Endocrinology*, 1939, **25**, 57.

[†] The synergist was prepared from sheep pituitaries. The unit was defined as the amount of synergist which in combination with 15 I.U. of chorionic gonadotrophin will cause a 5-fold increase in ovarian weight over that of the uninjected controls. Human chorionic gonadotrophin was present in an amount equivalent to about 0.25 I.U. per mg (22.2 units) of synergist. The synergist was kindly furnished by Dr. E. Schwenk of the Schering Corp., Bloomfield, N.J.

⁵ Cutuly, E., *Endocrinology*, 1942, **31**, 13.

⁶ Kupperman, H. S., and Greenblatt, R. B., *South. Med. J.*, 1946, **39**, 158.

[‡] That amount which produced positive ovary hyperemia reactions in approximately 75% of 5 or more rats.